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Future for unprofessional societies

One of the curious attributes of the English speaking world is its rich endowment of societies dedicated to advancement of science. The oldest among them have their roots at the end of what the historians call the Age of Improvement, the period spanning the end of the eighteenth century and the beginning of the industrial revolution. People all over the world then imagined prosperity could indeed be wrung from the careful application of technology and that profound social transformation might thus be brought about. The Napoleonic War appeared not to stifle optimists in Britain, while the haunting social dilemmas of the mid-nineteenth century had not been foreseen. What more natural then that people of goodwill should band together in societies for the development of science and technology? The result, still apparent, was the creation of a host of informal scientific societies as different as the Royal Institution (in London) and the American Philosophical Society (in Philadelphia) on the one hand and the British and American Associations for the Advancement of Science on the other. Although, with the passage of time, that part of the original role of these societies concerned with the communication of original science has now been taken over by the professional disciplinary societies that abound, many of them continue and even flourish. Why should this be and what role do they have to play in the decades ahead?

These questions are prompted by the arrival of the season in which the societies for the advancement of science hold their annual conferences. The British Association is meeting this week in the city of Salford (often wrongly called the slummy part of Manchester; the two cities, although contiguous, are constitutionally independent). The American Association will be meeting next January in Toronto, thereby demonstrating that the days have long since gone when members of the British Association would take to ships to hold their annual meetings in distant parts of the British Commonwealth. Instead, the example of the British Association has spawned similar organizations in India, Australasia and South Africa. In the next few months these informal societies will also be holding their annual meetings, somewhat formless gatherings attended by a few thousand people (but the organizers always hope for even more). The perpetuation of these ceremonies, even though they play no significant part in the exchange of information between professional scientists, is a sign that they must serve some purpose. What can that be? How might it be strengthened?

Casual observation, as at Salford this week, suggests several entirely beneficial functions of the unprofessional societies, of which the chief is that they are genuinely unprofessional. They provide the opportunity for people with diverse interests in science to hob-nob with people straight from the laboratory, and thus to learn a little more directly than would otherwise be possible what is happening at at least some parts of what Vannevar Bush called "the endless frontier". To be sure the frontier is covered only patchily at these huge gatherings, which also unhappily provide opportunities for the announcement of "discoveries" that would not filter through the usual refereeing processes by which articles in the scientific journals are judged. On balance, however, the opportunity to learn about science from at least some horses' mouths is much valued by those who attend the meetings and is also a wider public service. Many of the participants are people who teach in schools, or who are similarly isolated from the part of the scientific profession in which they learn their trade; nobody will dispute the importance of any means that helps such people to stay in touch. In recent years, all

the unprofessional societies, but the British Association in particular, have also found that young people — students and would-be students — have been the most lively participants at annual meetings. This is yet another sign that the unprofessional societies perform a valuable social function. The suggestion that the annual meetings might also lead to more general enlightenment through the general press is now less true than in the past; most newspapers regard their responsibility with continual seriousness and do not require such meetings to maintain their awareness. Even so, the unprofessional societies obviously render a service both to their members and, in some ill defined way, to the larger public. Why not, in the circumstances, let them get on with what they consider their business in the way they think best?

Unfortunately, that is too much to ask. To the extent that the unprofessional societies ask that public attention should be paid to what they do, public interest in how they set about their work is unavoidable. The case of the British Association is especially difficult, for since the Second World War, the Association has been living from hand to mouth, badly equipped for the job it seeks to do, too dependent on the goodwill and generosity of the cities (and their universities) in which it seeks to hold its annual meetings and on industrial companies and local authorities for the funds to support its regional activities and, most recently, on public funds (administered by the Royal Society) for the cost of running its tiny central office. On the half-a-dozen occasions in the past three decades, on which the cry of threatened bankruptcy has been heard, the Council of the British Association has resolved that radical changes of some kind must be made. And then, perhaps just too soon, funds have appeared from somewhere and have ensured that the changes made are neither radical nor, as it happens, effective. The result is that the British Association stumbles on, from one annual meeting to the next (or, with luck, the next but one) with a part-time Chief Executive Officer and an organization designed to ensure that decisions are made only slowly and half heartedly.

On the face of things, at least, the American Association for the Advancement of Science (AAAS) is in much better shape. Over the years, it has become a successful publisher, first of the distinguished scientific journal *Science* (whose 100th anniversary falls this year) but more recently of the popular journal, *Science 80*. With such a sheet anchor, the AAAS can provide for its own housekeeping and is even strong enough to compete for grants from federal agencies to enquire into matters as different as the renewal of the school science curriculum and the ethical problems (such as they may be) of new techniques in biology and medicine. With this financial symbiosis between its publishing and its public service the AAAS is relatively immune from outside criticism. Yet the organization is not nearly as successful as it might be. Like its British equivalent, the American association is hamstrung by the way in which its organization is built around a notion that there should be different sections of the society for the various disciplines that are from time to time invented. There is, of course, nothing wrong with the concept of disciplines within science but it is especially incongruous that disciplinary interests should be so firmly entrenched in societies claiming that they are essentially interdisciplinary. The fact that the officers of the hosts of sectional committees of the AAAS are elected publically, not nominated by taps on the shoulders from old chums in the British way, does not make the American organization more flexible, but the opposite. The outcome in both cases is that the societies for the advancement of science are usually too slow off the mark in



causes that might help to advance science, too inhibited (by their constitution) even from having views on many important matters and yet, paradoxically, whimsical — too much inclined to react unpredictably to changing circumstances and changing needs.

This complaint, harsh though it may seem to the devoted people who work hard at making the unprofessional societies function, is, nevertheless, quite fair. For what, in the past few years, have these societies done to help resolve some of the important problems in their declared fields of interest? Issues such as the hazards (if any) of genetic manipulation, public policy on nuclear power and even public policy towards the universities, may have been mentioned at the annual meetings of the British and American societies, but neither organization have used the diversity of its membership hammer out possible solutions. Providing a forum for opposing views on such issues is much easier, but also much less valuable, than attempting to resolve conflicts of opinion in ways that will carry conviction in and outside the scientific profession. Providing opportunities at the annual meetings for well known people to put their well known

views on grand subjects such as "Energy", "Population" or "Food" is similarly a largely unproductive enterprise. So how should the unprofessional societies put their houses in order? The most urgent need is that they find a way of tackling problems that at present impede the advancement of science — for one example see below. Organizations that cannot constitutionally have a view on issues such as the organization of research or the safety of nuclear power (and which may not even have studied them) can no longer claim to assist the advancement of science. The unprofessional societies have also been known to make links with their unprofessional constituencies — school teachers, industrial scientists and the like; this, however, is a task that could and should be tackled. (Both the Americans and the British societies will protest that many of their members are teachers, but that is not the same thing.) To accomplish these and other goals will undoubtedly require that the organizations themselves should change, perhaps substantially, but there is no reason to suppose that the change need be so drastic that the valuable aspects of what they do at present would be jeopardized.

Public-key cryptography muddle

The National Science Foundation and the National Security Agency in the United States are making a frightful muddle for themselves by their new policies on academic research in cryptography, made public last week. This is not the first time that there has been trouble between academic cryptographers and the United States Intelligence Agencies. Four years ago there was a minor storm when it turned out that an official of the security agency had written to the (American) Institute of Electrical and Electronic Engineers suggesting that articles on the design of new codes should not be published in the interests of security. Eventually, the row died down only when the official concerned was said to have written in a personal capacity. The latest development is more serious. The National Science Foundation and the National Security Agency have apparently reached an understanding on the processing of future research grant applications in cryptography. The National Security Agency will, it seems, now see all applications in the field that may be sent to the National Science Foundation and will, if it thinks fit, back good projects with its own funds. One consequence may be that more funds are available for research cryptography, which might lift the spirits of the academics in this field. Another is that the National Security Agency may attach to research grants the condition that the results should not be published without prior agreement from the intelligence people, a prospect that will dismay academics.

Is all this, then, a sinister plot to muzzle scientific research in the absence of legal instruments or 'classified' data as in the years immediately after the war? This is one reading of the new arrangement. Whatever the objectives of the National Security Agency the most likely consequence of the Washington agreement will be to make a monkey of the two partners, the National Science Foundation and the National Security Agency. The pace of academic research in this novel field is unlikely to be impeded. Publication, it is hoped, will continue without hindrance. And nobody's national security will be endangered.

What offends the intelligence people is the whole concept of public-key cryptography. They earnestly wish that it had never been invented. The notion is simply that of an unsymmetrical coding system, one in which it is not possible to infer the key for decoding a message in cypher from a knowledge of the rule by which coded messages are themselves produced from ordinary text. Consequently, with these novel codes, instructions for turning messages into codes could be published in the newspapers and it would, nevertheless, be impossible for somebody intercepting a coded message to decipher its meaning. For most people, the difficulty is that of understanding how there can be

codes whose writing and reading keys cannot instantly be inferred one from the other. The demonstration theorem required to dispell this scepticism has now been amply proved, most conspicuously by Professor L. J. Adelman of the Massachusetts Institute of Technology. The feasibility of the new coding system depends on the power of their computers now in service, which can use exceedingly complicated rules to turn ordinary text into coded messages. The basis of public-key cryptography is that the unpublished reading key cannot be inferred from the publishable writing key except with such a gigantic commitment of computer power that nobody in his senses would attempt the job.

Plainly, the National Security Agency is unconvinced. How can it be, these men in dark glasses whisper to each other, that a person can write in a code but be unable to decipher his own coded message? Somebody, they suspect, is pulling their legs. And they also appear to have forgotten that unsymmetrical coding systems, like those now being developed, are precisely the instruments needed to make commercial as well as military computer systems private and immune to espionage. Hitherto, there has been no means by which, for example, banks using public telex systems to transmit confidential information can have done so except by the old-fashioned methods of classical cryptography in which the intended recipient of a message is first provided with a secret code book. With the new codes, in principle at least, the writing key can be made freely available and the recipient of coded messages, the only one with access to the reading key, will be the only one able to decipher. The same techniques can and no doubt will be used to safeguard the privacy of personal data stored in central computing systems. The commercial importance of developments such as these is so self evident and so great that now that the potential value of public-key cryptography is appreciated, nothing will prevent the commercial computer companies from working hard towards the development of practical systems. Even if funds for research are not forthcoming from the National Science Foundation, there will no doubt be eager sponsors for academic research in the field of cryptography among the potential commercial users. For many academics however, the commercial sponsorship of research may be as irksome as the possibility that publication could be restricted — as the National Security Agency may choose to do. In practice, however, the sums of money needed in these fields are not large. The importance of free publication is self evident; quite apart from the intrinsic value of the new coded system, this is also a time when interest in the new field of cryptography needs to be stimulated. Is this not eminently a field in which private foundations should step in?

Carter pledges support for research

Washington

Reversing its policies of only a few months ago, the Carter administration has pledged that, if the President is re-elected in November, it will provide sufficient extra funds for basic research to guarantee a real growth of three per cent in both 1981 and 1982.

This would be achieved by requesting from Congress an additional \$600 million over the budget request already submitted for the fiscal year 1981 and which it had previously been proposed to request for 1982. The net result, according to Dr Frank Press, director of the Office of Science and Technology Policy (OSTP), will be to generate a real growth in basic research of about 11 per cent between 1978 and 1982.

The proposal to increase support for scientific research came as part of a general package of measures proposed by President Carter last Thursday, designed to revitalize the US economy through stimulating private and public investment.

Eschewing the major cuts in private taxation that have already been proposed by Republican presidential candidate Ronald Reagan, President Carter is proposing a more specifically geared programme that would reduce the tax burden on certain parts of industry and provide selective support for others.

In a speech outlining his general proposals, President Carter said that it was important to promote technological advance through support for research since it provided a large proportion of productivity growth and also "can create literally millions of jobs in the years ahead."

According to Dr Press, the precise distribution of the extra funds — about \$225 million of which are expected to go for the extra support of basic research in the fiscal year 1981, which begins in October — will be discussed with leaders of the university and business research communities.

However the initiatives will include:

- Increased support for areas of "targeted" basic research with broad potential applications. Examples provided by Dr Press included the contributions of genetic research to agriculture, and the basic research necessary to support the next generation of microcomputers.
- Increased support for university-based and other "generic technology centres", proposed in last year's domestic policy review of industrial innovation, and already under active consideration in Congress.
- Greater collaboration between government and industry in promoting the long-term growth of particular industries. The model of the Co-operative Automotive Research Project, looking at basic problems in long-term automobile

engineering, may be applied to other areas. Possible similar projects under consideration include cooperation with the steel industry on developing new techniques for producing highly specialized forms of steel, and with the mineral extraction industry.

● An additional \$50 million will be provided to upgrade research and teaching equipment in universities. A recent report produced by the American Association of Universities showed that in the university sector, the average age of equipment was about twice that in the industrial sector. The new money, some of which was proposed in the President's original

January request as part of the National Science Foundation budget but cut out in the March review, will support what Dr Press calls a "long overdue" renovation of research facilities.

Research and development investment in the private sector will also benefit from the new tax incentives which President Carter said that he proposes to introduce next year. In particular, this speeds up and simplifies the depreciation allowance on investment for research and development equipment, part of a general effort to persuade industry to invest more of what it earns in new technology.

"We are really trying to do two things

Congress falls in line on 1981 funds

Washington

With an election coming up, and few pretending that a balanced budget remains either a feasible or a desirable goal, Congress has taken several actions in the past few weeks indicating a general acceptance of the strategy of the Carter administration in support of research.

Indeed in many cases congressional actions on the research budgets of federal agencies have restored funding to the level of the original budget request put forward by President Carter in January, before being cut back in the budget review in March. Recent actions include: **Physics:** Following an unsuccessful attempt by the House of Representatives appropriations committee to cut out any increase in support of basic energy sciences in the Department of Energy — which includes high energy and nuclear physics — the equivalent Senate subcommittee has proposed various increases.

In high energy physics, for example, where the administration had requested in March a total of \$354 million for operating, equipment and construction costs, the House committee had recommended cutting this to \$327 million. The full House, however, subsequently amended this to \$343 million; and the Senate subcommittee last week agreed to propose \$359 million, the original request in January.

A similar pattern of changes is seen for nuclear physics, where the administration had requested \$111 million, the House agreed to \$106 million, and the Senate subcommittee is proposing \$113 million.

Physicists are optimistic that the Senate's actions will avoid extra delays in the construction of Fermilab's energy-doubler, and any severe cut in operating expenditure next year, both of which had been widely feared. "No one thought we could come close to these figures," said one university lobbyist last week.

Biomedical research: Although its

largesse has not been as great as in previous years, the House of Representatives voted last week to raise the administration's proposed 1.7 per cent increase for basic research in the National Institutes of Health to 5.5 per cent, reaching a total of \$3,616 million.

The House accepted the administration's argument that a central feature of NIH support should be maintaining the level of competitive research grants at about 5,000 awards a year. However it disagreed that training grants should be cut back in favour of research support, and much of the money added to the NIH budget will go towards postgraduate support.

National Science Foundation: The House voted last month to provide the NSF with the full \$1,074 million that had been requested by the administration, although subsequently agreeing to a 2 per cent cut across the board for all the agencies listed in the composite appropriations bill which includes the NSF.

The Senate appropriations subcommittee has also accepted the administration's request recommending a budget of \$1,079 million. In addition to shuffling \$5 million from basic research to science education (for which the House has allocated an extra \$10 million), the Senate subcommittee is proposing to add an extra \$5 million to cover the increased fuel cost being experienced by the NSF's Antarctic research programme.

Department of Defense: So far, the House of Representatives has accepted most of the 20 per cent increase in defence support for basic research requested by the administration. However the equivalent Senate committee is still to act; and there are rumours that cuts of the order of \$30 to \$50 million — which would reduce the growth rate to the expected rate of inflation — are being considered.

David Dickson

with this extra money," said one OSTP official. "Firstly to help underpin the general research base of the private sector. And secondly to support research, orientated towards the products and processes that are likely to be in place in the next few years."

According to Dr Press, the commitment to additional funds will "restore and go beyond the cutbacks proposed last March, which appeared to wipe out any hope of a real growth in support for basic research in the immediate future".

So far the scientific community has given the announcement a cautious welcome, but is waiting until more specific details are announced in the President's budget request next January — if he is re-elected — before passing judgement.

Although the total amount of funds may be small in comparison with the total US research and development effort, their effectiveness will depend on the way they are packaged. According to Dr Jack Crowley of the Association of American Universities "if they are willing to make some difficult decisions and focus funds in high priority areas, then they could achieve quite a bit."

Little direct reference has been made to research funding in the platform statements of the two main political parties. However the Republican statement does refer to the need to increase support for research and development as part of a general effort to increase US military strength.

David Dickson

NIH funding

Congress control

Washington

Despite intense opposition from the biomedical research community, the House of Representatives last week passed a bill which would, for the first time, give it direct responsibility for the budgets and research programmes of nine out of the eleven National Institutes of Health.

At present only two of the institutes — the National Cancer Institute and the National Heart, Lung and Blood Institute — are covered by specific authorizing legislation. The others fall within the broad responsibilities of the Department of Health and Human Resources.

The new bill sets generous budget ceilings for the various institutes, each of which would have its budget authorized by Congress every three years. Overall, the bill recommends a 27 per cent increase in the NIH budget in 1981 to a total of \$4,076 million, almost twice the budget increase proposed in the appropriations bill which was also passed last week (see page 3).

However, the implications of the legislation are that Congress will be in a position to exert more direct control over the research programmes of the nine institutes which currently are not controlled by

separate legislation. And it is this which has given rise to strong opposition from universities, medical schools and professional associations.

Writing in the *Washington Post* on 25 August, for example, two days before the vote took place on the bill, the past four directors of NIH stated "We are convinced that a long-term federal commitment to research is far and away best served by permanent authority of the type contained in . . . the Public Health Service Act."

The bill has been opposed by the American Association of Medical Colleges, the Association of Professors of Medicine, and the Joint Health Committee of the Association of American Universities. It has also been the source of fierce controversy within the administration. Initially it was opposed by Health Secretary Mrs Pat Harris, who argued that existing legislation was adequate to ensure a high standard of medically important research. Subsequently, however, after various modifications to the bill, the administration agreed to support the new legislation.

NIH administrators, who had previously been among those in opposition, were instructed to hold to the party line (*Nature*, 22 May 1980). And when the bill was introduced last week by Representative Henry Waxman, chairman of the health and environment subcommittee of the House Interstate and Foreign Commerce Committee, he said that both Dr Donald Fredrickson, director of NIH, and Dr Julius Richmond, the Surgeon General, had written giving their support.

Scientists fear that, by giving Congress access to detailed decisions on research priorities, the process could become over-politicized, encouraging short-sighted mandates to explore applied uses of research at the expense of basic research. However, during last week's debate on the bill, a number of congressmen spoke of the need to be in close contact with NIH, programmes.

Despite an intense lobbying campaign by university and medical school scientists, the proposed legislation was passed in the House by 292 votes to 48. It must now be reconciled with a counterpart bill which has been approved by the Senate, promoted by Senator Edward Kennedy.

This does not create authorizing legislation for all NIH institutes, but mandates the creation of a Presidential Commission on Biomedical Research with broad responsibilities to oversee medical research priorities.

David Dickson

Soviet academics

Wasted talent

Soviet planning is still not making sufficient use of the academic potential of the country, according to V. Kirillov-Ugryumov, Chairman of the Higher Attestation Commission (*Vysshaya*

Attestatsionnaya Komissiya—VAK) of the Council of Ministers of the USSR. In a *Pravda* article reviewing the work of VAK over the last five years, he pointed out shortcomings not only in VAK's work of awarding higher degrees, but also in the use made of the content of theses. The annual surveys, provided by VAK, of the content of theses and research projects, are not being utilized by the ministries and planning bodies concerned, and the recommendations made to the State Publishing Commission concerning the publication of the most interesting and significant theses are not being implemented. All this, he says, hinders the implementation of research results in the economy. The problem, moreover, is a particularly pressing one now that the country is on the threshold of the Twenty-Sixth Party Congress which will approve the directives for the next five-year plan.

Not, however, that VAK itself escapes without criticism. Six years ago the Central Committee of the Communist Party of the Soviet Union (CPSU) and the Council of Ministers of the USSR approved a resolution which overhauled the whole system of awarding higher degrees, in particular setting up a network of expert councils to assess theses submitted to them. Not all these councils, said Kirillov-Ugryumov, are functioning properly. In 1979 the presidium of VAK had to return no less than 256 works to the relevant councils since the reports on them were "insufficiently clear or well-grounded". Occasionally "ill-will" is creeping into the work of the councils, interfering with objective scientific assessment, and creating a situation resembling a court of law. Kirillov-Ugryumov goes on to decry "subjectivism and red-tape at any stage of the attestation process".

Nowhere in his article does Kirillov-Ugryumov mention a clause in the 1976 Resolution which many Soviet academics found disturbing — the stipulation that the postulants must satisfy VAK not only with the worth of their theses but also with their political record. Indeed, his criticism that certain expert councils have been letting "weak theses" through, and that steps must be taken to remedy this may well be a veiled criticism of councils which look over-leniently on the mediocre efforts of the scions of high party officials. However, a *samizdat* essay by Grigori Freiman recently republished in the West, "*It seems I am a Jew*" contains what is claimed to be a stenographic transcript of an "expert council" meeting, in which remarkable feats of dialectic are produced to find some reason, other than the postulant's Jewishness, to reject a thesis which all participants know in advance cannot be accepted. If Freiman is to be believed — and at the time of writing he was a Professor of the Kalinin State University, and a member of the CPSU — the political clause is apparently being applied under some other guise.

Vera Rich

European coal industry

Conversion plans

Plans for commercial coal gasification plants in West Germany will be complete by December, and liquefaction plants by next autumn, the Federal Ministry for Science and Technology has announced. But because German coal is very deep and very expensive, the intention is more to take a lead in the world market for such technology, than to make much use of it in Germany. So, although the construction of 14 coal refining pilot plants costing £3 billion is under study, Research Minister Volker Hauß said last week that oil and gas derived from coal would supply only 3 per cent of Germany's oil and gas needs in 1990.

Germany was forced to develop gasification and liquefaction techniques during the Second World War to provide the country with fuel, and although the technology then became uneconomic compared with cheap Middle East oil, the industry survived through massive exports to South Africa. After a lull in the sixties, German efforts were revived by the oil price rise of 1973. The United States have since spent more on developing new approaches and principles, but Germany appears to be taking a lead with real plant. German spending on research and development of new coal technologies (including remote extraction) is high, taking the lion's share of the non-nuclear energy research programme, which in turn costs some £230 million a year including industrial participation.

In the United Kingdom, investment is nowhere near so great. British Gas has just placed the contract for one pilot gasifier, using a mix of technologies and capable of converting coal dust and lump coal, at a cost of around £14 million; but this can be set against British Gas profits of some £426 million last year alone on retailing North Sea gas. The National Coal Board (NCB) has less to spend, but has recently recommended the construction of two pilot 1 tonne per hour liquefaction plants, one using the liquid solvent extraction process and the other "super-critical gas extraction"; the cost would be £50 million shared between the NCB, British Petroleum, and the Department of Energy.

However, according to an NCB scientist, the British technology is unique in aiming to produce nearly 100% conversion of the coal into high quality transport fuels, plus some liquid petroleum gas and synthetic natural gas. The technology could provide enough impetus to resist penetration of the British market by German or US technology, and might even provide export potential. The world market in sophisticated coal technologies is expected to reach billions of pounds a year in the 1990s, when oil prices rise to a level which would make coal competitive.

Robert Walgate

Hunter turned hunted

According to the Royal Society for the Protection of Birds at least 17 young peregrine falcons were stolen from UK nests this year. With peregrines selling to falconers at up to £1,500 each, nest robbery threatens to reverse the trend towards restoration of the UK breeding population which was severely affected by organochlorine pesticides twenty years ago. Present legislation prohibits the taking but not the keeping of wild birds. But the UK government has just announced plans to legislate against keeping also; it would require all birds of prey in legal captivity to be registered and ringed.



Third World energy

World Bank aid

Washington

Substantial new funds for energy research in developing countries could be one outcome of the proposals announced by the World Bank in Washington last week to help these countries meet their rapidly escalating fuel bills.

The main focus of the Bank's proposals would be the setting up of a new capital investment fund, which would raise money from private banks and lend it to Third World countries for energy projects ranging from nuclear power stations to conservation programmes.

At the same time, however, ideas for sponsoring relevant lines of research are being discussed. One possibility is that an independent fund or institute for research in renewable energy technologies could be established, perhaps taking a cue from the international group of agricultural research centres whose support the Bank currently coordinates.

In addition to the practical problems caused by energy shortages, World Bank officials have been worried that rising energy costs have made it increasingly difficult for many developing countries to pay off their international debts.

In Brazil, for example, the cost of imported petroleum rose from 12 per cent of export earnings in 1973 to 50 per cent last year; in India the increase was from 22 to 60 per cent over the same period.

The result, according to the *World Development Report, 1980* published by the Bank two weeks ago, is that major oil-producing countries will run current account surpluses of about \$110 billion in 1980, while the oil-importing, developing countries will have a combined deficit of more than \$60 billion. Efforts have been under way for some time to recycle the surpluses to ease the burden on developing countries. The political importance of this

was agreed at the economic summit of western leaders in Venice in June.

The World Bank has been watching the situation closely since 1977 and has started making loans for energy projects. This year, following particularly steep price rises, the Bank's president Mr Robert MacNamara asked his staff for a special report on what additional steps might be taken. This report, including sections on conservation and renewable energy sources, was recently made public.

Its main proposal is that the Bank should set up a special affiliate to raise sufficient money on the private capital market to almost double the \$13 billion in loans which the Bank itself intends to make over the next five years on energy projects. This should be sufficient to seed an additional \$75 billion in bilateral loans, and concessional terms might be arranged for the poorer countries.

The Bank says that many of the projects it already finances show a high return on investment, sometimes up to 30 or 50 per cent. But in many countries difficulty in raising capital has resulted in only superficial efforts to seek oil, and therefore "not enough research and money have been put into the effort to establish whether there are smaller deposits that could make important contributions to their own energy supplies".

Reversing its earlier opinion, the Bank says that it now agrees that conservation programmes offer a substantial potential for cutting back energy needs.

The proposals will be discussed when the World Bank and the International Monetary Fund meet in Washington later this month. However, it is generally believed that Mr MacNamara already has informal commitments that at least part of the money can be raised.

The precise nature of the fund, however, is still open, and so too are details of the way in which money might be used to finance research — although according to World Bank officials one of the biggest needs is the collection and evaluation of performance data about new energy

technologies that have already been developed.

The World Bank's report refers to the need to strengthen the national energy research programmes of developing countries, in particular their capacity to adapt developments made elsewhere. And it also says that attention should be given "to the possibility of organizing international programmes on specific renewable energy technologies".

David Dickson

Meat substitutes

Fungal food

Ranks Hovis McDougall (RHM) have received qualified approval from the UK Ministry of Agriculture Fisheries and Food (MAFF) to market, for human consumption, the somewhat processed mycelia of a *Fusarium* fungus. The company has now taken the decision in principle to test whether there is a sufficient market to make a commercial success of the processed mycelia, which goes by the name of mycoprotein.

A pilot plant at RHM Research in High Wycombe, Buckinghamshire, has already produced up to 100 tons per annum of the processed mycelia, sufficient to carry out extensive toxicological and nutritional tests. These have shown that mycoprotein can be fed without ill effects to generations of laboratory animals. It has also passed a number of short-term tests on human volunteers. In nutritional terms mycoprotein compares well with meat, for which it might be a substitute. On a dry weight basis it contains 45 per cent protein — with an acceptable amino acid composition — and has a low fat (10–15 per cent, mostly unsaturated) and cholesterol content. There is also no doubt that it can be made palatable. The only questions now are whether or not its high fibre content (20–25 per cent) is as beneficial as current advocates of fibre would have it, and whether it will meet consumer resistance.

The fungus from which mycoprotein is produced is the A35 strain of *Fusarium graminearum*, a strain which has lost the pathogenicity to plants possessed by its relatives. It is grown in continuous culture at 30°C in 1,300-litre tanks with glucose-syrup as the carbon source and ammonia as the source of nitrogen. A key process follows in which the mycelia are immediately heated up to 64°C for 20 minutes. That treatment kills the mycelia,

inactivates their proteolytic enzymes, so that there is no breakdown of protein, but allows the fungal ribonucleases to degrade nucleic acids into products which can be washed out of the cells. In that way the nucleic acid content of the mycelia is reduced from 10 per cent to below the acceptable upper limit of 1 per cent.

Furthermore the rapid heat treatment of the mycelia serves to reduce turgor with the result that mycoprotein has a texture that is not dissimilar to meat fibre. With the addition of flavouring and colouring, it can be made into a passable imitation of fish, chicken, veal or ham. Without any additions it is an unpalatable, colourless, tasteless product.

Human trials of mycoprotein have been very limited so far. Two small-scale and short-term nutritional studies have been carried out by Professor N. S. Scrimshaw at the Massachusetts Institute of Technology and some larger but less rigorous tests have been completed by RHM. The only problem that has emerged is a single case of an allergic reaction to mycoprotein which RHM claim is about par for the course.

On the basis of the human and animal tests MAFF has given permission to RHM to market mycoprotein on a limited scale once a specification has been agreed upon. It has also asked for further animal work to assess the effects of mycoprotein on mineral balance. There are two concerns in that respect. The first is that mycoprotein is lacking in the minerals, particularly iron and zinc, contained in meat. And the second is that the high fibre content of mycoprotein will lead to the retention in the intestine of dietary minerals from other sources — hence a recent recommendation that soya protein for human consumption be fortified with iron.

Mycoprotein is the first of its kind. Several other microorganisms are grown and processed world-wide to provide protein supplements to animal feed, but none for human consumption. Also, as Dr J. Edelman, Director of Research at RHM, is at pains to point out, most of the animal feed supplements are the product of bacteria growing on hydrocarbons, and hence classifiable as single cell protein, whereas mycoprotein is produced by a multicellular fungus growing on glucose.

The main question mark still hanging over the future of mycoprotein is whether or not the public will buy it. When the project was started RHM had their eye on the protein gap that was believed to exist in

many developing countries. That gap has since been declared a myth. Consequently RHM now have their eye on the UK convenience-food market (plans to seek approval for sale in the US have been put on ice because of the attitude of the Food and Drug Administration). To break into that market with mycoprotein will be no easy matter. Psychological barriers to new foods may be surmountable if the price is right but mycoprotein can only be cheap if produced in large enough quantity. Ten thousand tons per annum (0.25 per cent of annual UK meat consumption) would be realistic for that purpose, claims Dr Edelman; one hundred thousand tons would be "a bonanza" for the "nylon of the food trade".

Peter Newmark

Culture collections

Fears for fungi

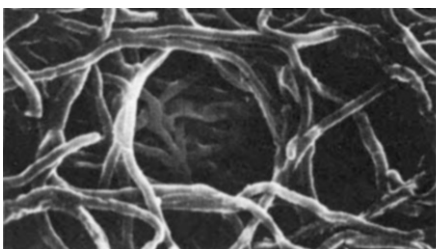
The effectiveness of Britain's main collection of non-pathogenic microfungi could be seriously reduced from March next year if £20,000 annual support cannot be found in the meantime. The collection is currently funded to the tune of approximately £60,000 per annum by the Commonwealth Mycological Institute (CMI), which houses it, and £20,000 by the Natural Environment Research Council (NERC), which has said that it will cease to pay its share early next year.

Similar problems were experienced earlier this year by the National Collection of Yeast Cultures (see *Nature*, 24 January), as a result of which an inter-Research Council committee was set up with the aim of observing and monitoring the maintenance of culture collections. The problems of the microfungi collection are the first to be investigated by the new committee. Next week, members of the committee will visit the CMI to make detailed recommendations on how the collection should be continued and whether alternative funds are needed. It will then start looking for new sources of money.

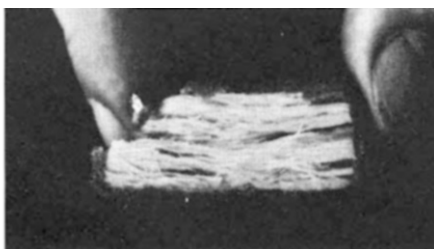
The difficulties with both the yeast and microfungi collections have arisen because they have never been permanently funded. NERC took over the bill for the microfungi collection ten years ago from the then Ministry of Trade and Industry on a temporary basis. The grant was designed to rise with inflation up to a ceiling of £20,000, a level that was reached several years ago. According to the curator of the microfungi collection, Dr Agnes Onions, the collection is already run on a shoestring and if the NERC funds are not replaced it will be impossible to fulfill all the functions of a national collection.

The collection is the main source of non-pathogenic microfungi in Britain for industry, research and educational establishments.

Judy Redfearn



Fusarium mycelia before



... and after

NEWS AND VIEWS

Active experiments in space plasmas

From Michael J. Rycroft

SPACE physicists have in recent years been able to plan and conduct 'controlled' experiments in the ionospheric and magnetospheric plasmas. In the experiments, the medium near the space vehicle is deliberately perturbed by the injection of a signal of known characteristics. The signal may be in the form of energetic charged particles; for example, electrons with a particular energy, power and pulse duration injected with an electron gun. Alternatively, the signal may be in the form of electromagnetic waves; an example is the transmitter at Siple Station, Antarctica, injecting VLF waves of known frequency, amplitude and duration. The propagation of the signals can be detected enabling the medium remote from the space vehicle to be investigated.

As a recent conference* showed, the situation is not always as simple as that initially conceived by the experimenters. Soviet Zarnitza and Franco-Soviet Araks experiments of 1975 and recent US and Canadian experiments show that the operation of a high power electron gun aboard a rocket in the upper atmosphere causes complex processes to occur. A Beam Plasma Discharge (BDP) is often initiated; in this, the electron beam develops an instability in which ionospheric electrons are accelerated by plasma (Langmuir) oscillations. If heating to energies > 15 eV occurs, collisions with neutral molecules produce secondary electrons. If the neutral gas density, including the local increase due to outgassing from the rocket body, is high enough, this process cascades. Then a hot plasma halo is formed around the rocket; this is cigar-shaped and aligned along the geomagnetic field. Detectable by a low light level television system, the halo can have a luminosity equivalent to that of a fifth magnitude star. By providing the neutralization current, the enhanced plasma density in this region prevents the rocket potential rising as high as might otherwise have been expected for large electron beam currents.

This halo region is a source of HF (up to ~ 50 MHz) radio waves, at $\sim 2 \times 10^{-20}$ $\text{Wm}^{-2}\text{Hz}^{-1}$. These electromagnetic waves are believed to be produced by conversion from electrostatic waves at the large gradients of plasma density existing at the edges of this region. Swept frequency studies show that the radiation is generated preferentially at the electron cyclotron frequency and at the electron plasma frequency, and also at the upper hybrid resonance frequency which is their Pythagorean sum. Radar reflections from the region are also observed. Whistler mode radiation is detected, as are bursts of noise at the lower hybrid resonance frequency which is inversely proportional to the square root of the mean ionic mass of the plasma.

Recently, Norwegian groups have successfully used mother-daughter payloads, with a pulsed electron accelerator being mounted on the daughter and the mother bearing a number of diagnostic instruments. The dimensions of the heated region are found to be two electron Larmor radii across the geomagnetic field and several times that along it. Noise bursts are observed to be generated, not only near the lower hybrid resonance frequency but also at lower frequencies, when the rocket crosses an auroral flux tube in the presence of precipitating energetic electrons. The waves are believed to be produced locally in the region perturbed by the beam and the neutralizing return current. In this issue of *Nature* (p.15) Gough *et al.* present observations, associated with the operation of the electron gun, of 0.8–1.0 MHz electrostatic waves and 8–10 KeV auroral electrons bunched at the same frequency. Waves are excited by the beam when it is injected into the ionospheric plasma; these in turn bunch the electrons in the beam.

A new development is in the use of huge vacuum chambers, such as the 12 m by 26 m chamber at the NASA Johnson Space Center, for laboratory investigation of the behaviour of electron guns before launch. Television recordings show that for low gun currents, the emitted electrons follow helical paths in the magnetic field. As the gun current is increased, a Beam Plasma Discharge is suddenly ignited. This is seen as a spatially broad beam, having a

wide distribution in energy and with considerable light output due to the increased plasma production rate. Radiation in the whistler mode and Bernstein modes is recorded, as is noise around the electron plasma frequency and the upper hybrid resonance frequency. Performing experiments related to space plasma physics in the laboratory is a very necessary adjunct to performing laboratory-type active experiments in space plasmas.

Operation of a caesium plasma generator simultaneously with the electron gun in the Araks experiment caused the upper frequency of the HF emissions to increase above 50 MHz. In the West German Project Porcupine, experiments have been carried out involving ~ 200 eV xenon plasma injection. The dynamics of the plasma jet and associated wave processes are studied using instruments on the main rocket and on sub-payloads ejected from it. Particularly interesting are plasma instabilities induced by the xenon injection; sometimes these manifest themselves as electrostatic wave bands whose frequency separation is the cyclotron frequency of protons, but sometimes it is half this frequency. These ion cyclotron harmonic waves, which are detected both as AC electric field and plasma density perturbations, extend in frequency up to at least the twentieth harmonic. The most intense signals are located close to the estimated lower hybrid resonance frequency; of course, this frequency is changed considerably by the ion injection.

With shaped-charge explosive devices aboard rockets, barium has been injected upwards at high velocity. After ionization by solar ultra-violet radiation, the ions move up the geomagnetic flux tubes. Using sensitive optical equipment on the ground, upwards acceleration has sometimes been observed to occur at a height of a few thousand km, presumably by the action of an electric field. Such an electric field would accelerate auroral electrons

A symposium on 'Active experiments in Space Plasmas' was held in Budapest from 11 to 13 June, 1980 as part of the 23rd meeting of COSPAR (the Committee on Space Research) established by the ICSU in 1958. Most of the invited review and contributed papers presented at the Symposium are to be published early in 1981 by Pergamon Press as a volume in the COSPAR Advances in Space Exploration series.

Michael J. Rycroft is Head of the Atmospheric Sciences Division, British Antarctic Survey, Natural Environment Research Council, Cambridge and was a member of the Symposium's Organising committee.

downwards.

Project Trigger was a Swedish and US experiment designed to test the response of the auroral ionosphere to the explosive release of a hot, dense caesium plasma. A transient electric field is observed, as are variations of the field aligned fluxes of charged particles from 10 eV to > 300 keV. It is concluded that the field-aligned currents associated with the electric field pulse and cloud conductivity gradient may be responsible for the observed electron acceleration in a manner similar to that leading to the formation of auroral arcs.

Disturbances to the ionosphere are also caused by the explosive release of water from rockets. The ionospheric electrons rapidly recombine, leaving a 'hole' in the ionosphere. Simultaneously the flux of primary auroral electrons decreases by two orders of magnitude, probably because of changes in the auroral 'circuit' caused by the local conductivity decrease. Similar holes in the ionosphere are introduced inadvertently by the highly reactive exhaust gases from rocket engines. The Saturn V vehicle, which launched Skylab in 1973, caused the ionospheric electron content to be halved. The launch, in 1979, of the HEAO-C satellite by an Atlas/Centaur rocket provided confirmation of the effect. The ionosphere was significantly depleted over a horizontal distance of 300 km for some hours.

Ion engine thrusters are now being developed. Using these, ions will be injected into the magnetosphere in the form of a dense, relatively cool plasma beam with a high drift speed. Their effects have not been thoroughly investigated, either observationally or theoretically. Such work is essential because, for example, plans are being made in the US to transfer a Solar Power Satellite, of $\sim 10^7$ kg, from low earth orbit to geosynchronous orbit using argon ion thrusters. This would deposit $\sim 10^6$ kg of 3.5 keV argon ions into the magnetosphere. The $\sim 10^{31}$ argon ions would equal the natural content of the magnetosphere, but their energy content would be about a thousand times larger than that present naturally. Such plans are therefore of considerable concern to magnetospheric physicists. It is desirable that preliminary experiments using ion thrusters in the magnetosphere be carried out as soon as possible.

A complementary type of active experiment is the injection of waves into the ionospheric or magnetospheric plasma. Since the launching of the Canadian top-side sounder satellites in 1962, plasma resonances have been stimulated by antennas within the natural space plasma. However, only since 1977, with the advent of the pairs of GEOS and ISEE satellites, and with the Japanese Jikiken (EXOS-B) satellite, have such resonances been studied in the magnetosphere and solar wind. Plasma frequency and upper hybrid resonance frequency signals enable plasmaspheric

electron density profiles to be derived; some of these clearly exhibit the plasma-pause with attendant fine structure. Bernstein mode electrostatic resonances, the so-called f_{Qn} resonances, plus resonances at harmonics of the electron cyclotron frequency are also observed. The effects of the drifting plasma and of its non-Maxwellian distribution have to be taken into account when interpreting these signals.

In recent years, controlled experiments on the magnetosphere have been carried out using coherent VLF electromagnetic waves injected from Siple Station, Antarctica which are ducted in the whistler mode from one hemisphere to the other. When radiating above a certain power (<1W–2kW), these signals exhibit growth rates ranging from 30dB s⁻¹–200 dB s⁻¹. The energy source here is the cyclotron motion of the van Allen radiation belt electrons near the equatorial plane just inside the plasmapause; in the interaction the electrons' pitch angles are reduced and they can be precipitated into the upper atmosphere. The amplified signals can trigger emissions whose frequency varies with time. Natural emissions are 'entrained', i.e. their frequency-time evolution is dramatically changed when their frequency comes within ~ 50 Hz of that of the coherent Siple signal. Sidebands, with growth evident only in the upper sideband, are generated. Growth of the Siple signals is suppressed either by a whistler mode echo of the signal or by a superposed broadband signal. Growth of natural noise is suppressed at frequencies just less than that of the Siple signal. Unducted signals from the Siple transmitter have been observed on the ISEE and Jikiken spacecraft. Triggering of emissions is evident when the satellite is in the magnetic Northern hemisphere, that is, after the Siple signal has crossed the equatorial plane of the plasmasphere. *In situ* wave-particle interaction studies now become possible, an exciting prospect indeed.

Radiation at harmonics of the fundamental frequency of the electrical power distribution (grid) systems of the industrialised world similarly penetrates the ionosphere and propagates in the whistler mode through the magnetosphere plasma. In principle it can interact with energetic electrons in much the same way as do the Siple signals. There is considerable evidence that it does in fact do so, though more observational studies are required. The significance of this 'pollution' of the magnetosphere remains to be quantified.

There is some controversy too as to whether the occurrence of chorus is affected by power line harmonic radiation. However, studies at Halley, Antarctica, which is geomagnetically conjugate to Newfoundland where there is a mixed 50 and 60 Hz power system, show that the intensity of noise of magnetospheric origin at 3.2 kHz is, on average, 0.7 dB less on

Sundays than on other days of the week. In a similar vein, but at very much lower frequencies, there is evidence from a century of data that geomagnetic activity is larger, by one standard deviation, at the weekends than during the week. Recent evidence shows that 1 pc pulsations of the geomagnetic field decrease when the DC operated San Francisco Bay Area Rapid Transit train system is operating. Could it be that the growth of ULF coherent waves, by a cyclotron resonance interaction with ions in the magnetosphere, is inhibited by ULF noise of man-made origin?

An HF transmitter, with an effective radiated power > 100 MW, has recently been constructed in northern Norway by West Germany. This powerful heater of the overhead ionosphere can modulate the temperature, and hence the conductivity, of the auroral ionosphere at frequencies between 0.2 and 5 kHz. In the presence of the auroral E-region electrojet, a signal at the frequency of the modulation is radiated. Signals with the appropriate whistler mode dispersion are recorded on the ground. A basically similar phenomenon, but with heating signals propagating obliquely from LF or MF Soviet transmitters, is responsible for the demodulation and radiation of 1 kHz timing pips, which can be received on the ground. More controlled experiments are required in this new field.

The present decade will see the first experiments carried out aboard Spacelab, to be launched by the Space Shuttle. The field of active experiments in space plasmas is well represented here. For example, Canada is proposing an MF/HF transmitter/receiver, with 0.5 kW peak power, using a dipole antenna of up to 300 m length tip-to-tip. Studies are planned of the transmitting antenna itself, ionospheric soundings, electromagnetic and electrostatic wave propagation in magnetoplasmas, parametric decay and plasma instabilities and nonlinearities. VLF wave injection is also proposed using the same antenna, the size being comparable with the whistler wavelength in the ionospheric medium. Waves can thus be injected over a range of wave normal angles (to the geomagnetic field) that are inaccessible from ground-based transmitters. The aim of such experiments is an increased understanding and control of magnetospheric wave-particle interaction mechanisms. Chemical releases, including water produced by fuel cells, from the shuttle are also planned. Finally, on the first Spacelab now scheduled for launch in 1983, Japanese, US and European groups are collaborating on intense (maximum 7.5 kV, 1.6A) electron injection experiments. These are designed to excite artificial aurorae, to study beam-plasma interactions in the ionosphere and to study charging of the Space Shuttle by operation of the gun and its neutralization by both plasma arcjets and neutral nitrogen plumes. □



100 years ago

A balloon ascent was made at Cherbourg, on the occasion of the *fête* given by the Municipality to M. Grévy, by MM. Perr and Capt. Gauthier. The general direction of the wind being from the land to the sea, a government steamer was sent out to secure the safety of the *aéronauts* if necessary. Before starting not less than thirty pilot-balloons were sent up to ascertain the superposition of the aerial currents. It was proved that at 40 metres the wind was blowing from the sea. After having travelled for more than an hour in the direction of Portsmouth the *aéronauts* opened their valve and returned safely

on shore. More than a hundred thousand spectators witnessed the experiment. The culminating point of the ascent was an altitude of 1,500 metres, where the travellers could see the English coast, the whole of the Isle of Wight, &c. The scenery is stated to have surpassed description. Some very curious observations were made on the colours of the sea. In the places where the water is very deep it looks quite inky, and the curves of level are so clearly manifested that they bear comparison with equidistance lines worked on ordnance maps. When travelling at so great an altitude ships can be detected with some difficulty; but smoke can be seen even when the smoke-producing steamer can hardly be perceived with the naked eye.

On the evening of July 20, about half-past eight o'clock a remarkable meteor, said to have resembled a comet,

apparently about twenty yards in length was observed at Vizimbaum and other places in India, traversing the sky from south to north, and remaining visible for about three-quarters of a minute, during which time the whole sky and country were brilliantly illuminated. The meteor then burst, and some time afterwards a loud sound like distant thunder, which lasted two minutes, was heard.

At a concert given every night in the garden of the Palais Royale, Paris, the orchestra is placed in the vicinity of the fountains which are illuminated by eight splendid Siemens lamps, which work admirably. Two other Siemens lamps have been placed in the shop of a jeweller in the Galleries, and the experiment may eventually lead to the lighting of the whole palace by the electric light.

from *Nature* 22, 26 August, 401, 1880

Maverick mitochondria

from a correspondent

LAST November, readers of these columns will recollect, the news broke that the genetic code, hitherto thought to be universal, was different in mitochondria. Now, new and startling revelations concerning the machinery that mitochondria use to translate the genetic code are described in three recent papers (*Proc. natn. Acad. Sci. U.S.A.* 77, 1980).

Mitochondria are organelles which exist inside eukaryotic cells where they perform the rites of oxidative phosphorylation. They possess their own DNA which contains genes for the enzymes they require for these rites as well as for their own ribosomal RNA and tRNA. To grasp the importance of the new findings we must first note that the 64 possible codons may be arranged in 16 families. Each family is defined by the first two bases of its codons so that, for instance, GUN, where *N* can be U, C, A or G defines the codons of the GU family. It is convenient to refer to the two codons of a family with a pyrimidine (U or C) in the third or 'wobble' position as the pyrimidine codons and the other two with A or G as the purine codons. In the standard genetic code, eight of the 16 families are unmixed: that is say all four codons in one family code for the same amino acid. In the mixed families several patterns of assignment exist; in six of them the pyrimidine codons signal one amino acid and the purine codons another or, in one family act as termination signals.

In the standard translational machinery, the codons interact one after another with tRNA molecules each bearing an appropriate amino acid. Each tRNA molecule has an anticodon of three bases that

interact in an anti-parallel manner with the codon forming three base pairs. The first two base pairings are of the standard Watson-Crick type but the third in the wobble position may be more variable. In this way an anticodon may read from one to three different codons of the same family according to the nature of the base in its wobble position. For instance, an anticodon with U in the wobble position may read both purine codons of a family and one with G may read both pyrimidine codons. With the rules of wobble pairing in mind, it can be shown that a minimum of 31 types of tRNA are required to read the standard genetic code.

The three new papers first of all extend and consolidate our knowledge of the deviations from the standard code that occur in mitochondria from *Neurospora crassa*. (Heckman *et al. Proc. natn. Acad. Sci. U.S.A.* 77, 3159; 1980), from mammals (Barrell *et al. Proc. natn. Acad. Sci. U.S.A.* 77, 3164; 1980) and from yeast (Bonitz *et al. Proc. natn. Acad. Sci. U.S.A.* 77, 3167; 1980). In mitochondria from all three species it now transpires that UGA, instead of acting as a termination signal, codes for tryptophan as does the other purine codon from the UG family. In the AU family the code differs from standard only in mammalian mitochondria where the pyrimidine codons signal isoleucine and the purine codons methionine. Yeast and *Neurospora* abide by the standard pattern in this family. A further change occurs in the unmixed CU family of yeast in which all four codons signal threonine instead of the standard leucine. Apart from these deviations, the

other codon assignments in mammals and yeast appear to be standard except for a suggestion that certain codons, normally used for arginine and which have not been detected in portions of the genome translated into protein, may be used as termination signals. In *Neurospora* no further deviants have been detected but the data are still incomplete. It may be noted in summary that the codes used by these three species not only differ from the standard but also differ among themselves. How many other deviations will show up in mitochondria from other species remains to be seen.

These observations, interesting though they may be, do not constitute the major bombshell delivered by these papers. As portents have been seen for some time it is disturbing that nobody has been able to find a full complement of 31 tRNA species in mitochondria, particularly since it has been shown that no extra tRNA is imported from the cytoplasm, at any rate in mammals.

Barrell *et al.* have now shown, by sequencing virtually the entire mitochondrial genome, that there are indeed only 23 tRNA genes in HeLa cell mitochondria. Similarly Bonitz *et al.* have found only 24 in yeast. An examination of the putative anticodons of these genes allows each to be assigned to a codon family. When this is done it is seen that eight are assigned to unmixed families. Invariably in mammals and in 7 cases in yeast these anticodons have a putative U in the wobble position. Since most of the 32 codons involved appear to be used to code for the

appropriate amino acid it would appear that in some way these tRNA species can read all four codons of their families by recognising the two non-wobble bases and ignoring the third — a mechanism that I will call super-wobble. This is clearly anomalous but reminiscent of Lagerkvist's two-out-of-three reading scheme (Lagerkvist *Proc. natn. Acad. Sci. U.S.A.* 75, 2759; 1978). The plot thickens when it is seen that eight further putative anticodons with G in the wobble position must be assigned, one each, to the remaining mixed families and six with a putative U to families requiring them. This makes it possible to read the mixed families according to standard wobble mechanisms.

A grave anomaly remains however. If an anticodon with U in the wobble position is able to read all four codons of an unmixed family, oblivious of the wobble base in the codon, how is it that the anticodons with a U in the third position are able to confine their attentions to the purine codons in the

mixed families? To put it another way: why does super-wobble only occur in the unmixed families?

A clue to the answer to this conundrum is to be found in the paper by Heckman *et al.* They show from determinations of the sequence of six tRNA species that the U in those anticodons which must not indulge in super-wobble are modified (precisely how is still unclear) whereas the U in those that may so indulge is not modified. Modification inhibits super-wobble it seems but it remains still to be seen how this is done. Maybe the small and specialised mitochondrial ribosomes play some role in arranging this distinction. Meanwhile two points should be noted: first, most cytoplasmic tRNA species with a U in the wobble position have it in a modified form and second, this clue to the mechanism of the distinction is based on work on *Neurospora*. We must await further developments to see if the U's in mitochondrial tRNA from yeast or mammals are appropriately modified. □

and metabolic products seem unlikely to provide the clues. Compartmentation or availability of hormones during particular receptive stages in seed development or germination are more likely to determine their regulatory functions: the precise stage of receptivity presumably depending upon the presence of the appropriate receptor proteins rather than the hormone level.

While control mechanisms of dormancy prove elusive, the mobilization by the embryo of stored protein and carbohydrate reserves at germination are demonstrably under hormonal control from the developing axis.

On seed imbibition in peas and beans, amylolytic or proteolytic activity in the cotyledons with subsequent hydrolysis of stored starch and protein is induced by the presence of the embryo axis (the radicle) or by addition of cytokinins; end-product inhibition or high osmotic concentration in the cotyledon are apparently not the reason for suppression of these enzymes in the absence of the axis. In lettuce, which stores mainly oligomannans, the radicle appears to transmit a stimulus to endosperm cells to produce β -mannanase with further breakdown of the mannans by a β -mannosidase induced in the cotyledons; the whole representing a linked and presumed hormonally regulated sequence. In mung bean cotyledon cells an endopeptidase synthesised on endoplasmic reticulum is transferred to the storage protein bodies during germination; the ensuing hydrolysis of these proteins illustrates the autophagic nature of these bodies and the central role of endoplasmic reticulum in enzyme synthesis. The lighter buoyant density of endoplasmic reticulum of aleurone cells during gibberellin-induced α -amylase production is therefore of considerable interest.

The physical and metabolic effects of seed hydration at germination are critical to seedling establishment. Restraints on water flow to the seed are imposed by the hydraulic properties of the seed-bed and the water energy level of the seed. Again, mathematical modelling based on experimental assays under different water availabilities can now provide predictions of field performance. Saline conditions appear to cause greater reductions of shoot than root growth. Accumulation of abscisic acid in the stressed cotyledons followed by preferential transfer to the shoot may account for this.

Maintaining the water content at a critical level for some while during seed hydration can, however, lead to more uniform germination and convey a greater resistance to sub-optimal temperatures. This is achieved by pretreatments which include an osmoticum such as polyethyleneglycol in the ambient imbibition medium prior to sowing. Such seeds can then be dehydrated and stored before

Probing the mysteries of seeds

from Daphne J. Osborne

SEEDS are remarkable things. Each is a precise package enclosing a dry, quiescent and differentiated embryo, with attendant food reserves. Add water and within minutes the seed can be activated to renewed metabolic growth.

Almost every aspect of seed physiology and biochemistry was covered in discussion at the recent International Seed Germination Conference*. Ecologists for example, were assessing germination opportunity by mathematical modelling to include climatic variables and competitive interactions. Dormancy and dispersal of seeds were considered as alternative winning stratagems, depending on the habitat. One interesting model was for 'small patch' survival. In many desert plants, anti-dispersal mechanisms (atelochoy) are winners. Seeds are held within the dried up plant until the rains so ensuring that they germinate in already successfully colonized sites.

Although a seed is said to have germinated when the root protrudes, there is no good definition in biochemical terms. The start of DNA replication could be the biochemical marker since imbibed but dormant seeds, although maintaining a continuous metabolic turnover, neither grow nor replicate their DNA and germinating embryos seem not to replicate their DNA until the radicle protrudes.

The depth of dormancy of seeds at shedding depends upon complex combinations of temperature, water availability and photoperiod during seed formation. Some seeds develop what is called secondary dormancy. This can happen if they remain imbibed under conditions in which they will not germinate. Those with a phytochrome-controlled dormancy, broken by exposure to red light may pass into secondary dormancy that is then independent of phytochrome control but broken by chilling. Some weed seeds develop a phytochrome-dependent secondary dormancy when they fail to germinate through lack of oxygen. This may account for successive bursts of germination observed in the field.

The question "do endogenous hormones control dormancy?" was a major talking point. Attempts to relate dormancy directly to either effects of hormone application, or to the levels of endogenous hormones in the seed proved unrewarding. For example, no relationship was established between abscisic acid and secondary dormancy and little understanding emerged for a mechanistic role for abscisic acid from the new, detailed knowledge of its metabolism during seed development and germination. Nor did there appear to be any obvious differences in abscisic acid biochemistry of dormant and non-dormant embryos which might account for their differences in germination. However, added abscisic acid does repress germination, so a role for abscisic acid is not excluded, even though studies of levels

*A Seminar on "Control Mechanisms of and during Seed Germination" sponsored by the Bat Sheva de Rothschild Foundation for the advancement of Science in Israel was held in the Hebrew University, Jerusalem, from March 16th to 29th. The proceedings will be published this year in Volume 28 of the *Israel Journal of Botany*.

sowing. But improvement does not always result. Secondary dormancy occurs if phytochrome controlled seeds are osmotically treated in the dark and there is a critical stage during imbibition before which the germinating seed is tolerant to subsequent dehydration and beyond which, removal of water results in cell death.

Desiccation tolerance of seeds is still poorly understood. It starts during seed formation when synthetic events are shut down. Polysomes are dissociated to monosomes with run-off of mRNA. Within minutes of rehydration at germination, polysomes are re-assembled suggesting that early protein synthesis could be initially independent of new RNA synthesis. But embryogenesis and germina-

tion are linked to changing protein patterns and in cotton cotyledons the maternal, constitutive and abscisic acid-induced protein products can be distinguished as well as their associated and different abundance classes of PolyA-rich RNA messages.

Ageing seeds in the dry state, apparently sustain increasing levels of single stranded breaks in nuclear DNA and an early desiccation-tolerant event at germination is the 'repair' of these lesions with likely restoration of genome fidelity. Such repair capability is important for proper seedling establishment for failure to repair may lead to impaired vigour and low levels of early protein synthesis.

None of the early events of germination are possible without an energy supply. ATP

levels are barely detectable in dry seeds. ATP production from inosine mono-phosphate or through an phosphoenol pyruvate-pyruvatekinase generating system are possible but measurements of energy charge favour oxidative phosphorylation. Differing mitochondrial activities may indeed be another factor controlling rates of early protein synthesis.

This meeting identified many problems, exposing the present lack of biochemical understanding of how desiccated cells survive, what makes them dormant and what breaks their dormancy. When knowledge in these areas becomes as far advanced as our current information on the biochemical events of early germination then another seminar will certainly be warranted. □

Bahamian Atlantis reconsidered

from Marshall McKusick and Eugene A. Shinn

DURING the late 1960s amateur explorers diving in shallow water found tabular limestone blocks which led them to repeatedly publish claims that Bimini Island in the Bahamas was the unsubmerged tip of the lost continent of Atlantis. In 1971, however, Harrison (*Nature* 230, 287) argued that the supposed stone walls or roads were in fact natural limestone and that some submarine structures described as pillars were hardened concrete originally stored in wooden barrels and dumped overboard in recent times at the harbour entrance.¹ Nevertheless many amateur explorers have ignored the scientific explanation and books and articles that perpetuate this Atlantis myth continue to appear.^{2,3} It is our contention that the persistence of the Bimini Island hoax must be explained in sociological rather than geological terms. There is in the US a vigorous cult dedicated to the mystic revelations of a native American prophet, Edgar Cayce (1877-1945), who wrote that lost Atlantis was the centre of all ancient civilization 10,000 years ago and that Bimini was part of that continent. Thus the Bimini affair has become a clash between scientific interpretation and religious dogma.

Geological studies have now gone beyond the 1971 geological reconnaissance of Harrison who wrote that a shell-hash gravel in shallow water was exposed and cemented during the most recent

emergence of the Bahamian Banks. In 1978 Shinn's study reported that the so-called 'man-made roadway' was actually submerged beachrock.⁴ On the Bimini beaches the high calcium content of the seawater allows the rapid formation of natural limestone beachrock — a fact demonstrated by glass shards, bottle caps, and other beach litter incorporated into the stone. Similar rapid formation of limestone has been shown in coral atolls in the Pacific where World WarII artefacts have been found in the matrix. Beachrock formed by the circulation and evaporation

of calcium laden seawater has the following characteristics: (1) tabular fractures, (2) keystone vugs, (3) a surface slope and internal laminations which tend to parallel the beach incline, (4) stratigraphic continuation of sedimentary layers from one tabular block to another, and (5) an overall geometry which reflects tidal zone origin, that is, a narrow band frequently extending a mile or more along the sea front.

Amateur enthusiasts have claimed that the Bimini blocks were quarried by ancient Atlanteans and laid out in an ancient 'Cyclopean, megalithic roadway' which

Beachrock forming on Heron Island, Australia displays just the same features as the 'Atlantis Road' in the Bahamas.



Marshall McKusick is Associate Professor of Anthropology, University of Iowa, Iowa City. Eugene Shinn is a member of the United States Geological Survey, Fisher Island Station, Miami Beach, Florida. His geological study at Bimini reported here was at his own expense and not part of any U.S.G.S. sponsored project.

involved using squares and rectangles of various sizes including some 15 feet across. However the limestone structures observed off Bimini in 15 feet of sea have all the features of natural beachrock.⁵ The limestone is in a narrow band overlying coral sand and extends for a considerable distance along a former shoreline. The so-called road's hairpin curve is not continuous and parallels the existing headland on shore. The tabular fractures are natural, and the original slope to the sea is present. A sample of 17 oriented cores obtained by Shinn and Tomkins has been examined with X-radiographs. Two areas of the formation were studied, and both show slope and uniform particle size, bedding planes and constant dip direction from one block to the next. If the stones had been quarried and relaid there is no reason to suppose bedding planes would carry stratigraphically from block to block. The sedimentary laminations clearly show that these were not randomly laid stones but a natural, relatively undisturbed formation.

Although under 15 feet of water, the beachrock is of recent geological origin. One ¹⁴C date on shell has already been published as 2,200 ± 150 years BP. Jerry J. Stipp (Radiocarbon Dating Lab., University of Miami) has run seven bulk samples from the cores as a class project and gives slightly older dates for the Bimini submerged beachrock.

Laboratory Number	¹⁴ C Years BP
UM - 1363	2745 ± 80
UM - 1364	2770 ± 80
UM - 1359	2780 ± 65
UM - 1365	2835 ± 70
UM - 1361	3355 ± 90
UM - 1360	3500 ± 80
UM - 1362	3510 ± 65

Testing of submerged features in Florida and one test on North Bimini Island shows that the sea level has risen at a rate of about one inch every 40 years for the past 5,000 years. This rate of submergence over 2,200 to 3,500 years would account for 5.58 to 7.22 feet of the 15 feet of sea observed over the beachrock. The remaining 7 to 9 feet of sea may be explained by the undermining of sand allowing the beachrock to gradually settle, an erosive process that is seen in various stages in many parts of the Caribbean. Harrison's map shows that the submerged beachrock is located on the most exposed shore subjected to substantial erosion during winter storms.

The Bimini mystery has been used to promote tourism — "scuba dive over the lost road of Atlantis" — and has been exploited commercially in other ways. During the 1970's an enormous number of books

describing ancient Atlantean, Phoenician, Egyptian, Viking, and other mysterious visitors to prehistoric America⁶ have been published by both the major and minor New York commercial presses. This science fiction disguised as historical explanation is popular with the reading public and is part of a contemporary fad. However, there is more to the Bimini-Atlantis hoax than commercialism. The mystic revelations of the American prophet Edgar Cayce have formed the basis of a successful religious cult with a national membership reported to be 18,000 and a staff of 110 at the Virginia Beach headquarters. The Cayce cult is a religion, and the revelations describe geological catastrophism as the explanation for Atlantis, the lost Pacific continent of Lemuria, and origin of civilization in such

mythical lands. The 'sinking' of Atlantis according to Cayce's revelations occurred 10,000 years ago, although the radio-carbon dates of the natural formation show it originated some 7,000 years later.

To explain the rejection of archaeological and geological evidence, it must be said that Bimini is just one of a growing number of archaeological 'shrines' which have become the object of cult worship. Pyramid worship is becoming more common, and in England itself, mystic cults centre upon the alleged religious powers of Stonehenge and other megalithic monuments. Whatever the explanation of these new religious movements, they have led to an unfortunate rejection of the scientific explanation of natural and physical phenomena among a significant segment of the reading public. □

Retrograde transport in axons

from Peter Banks

A novel technique has been developed by Fink and Gainer^{1,2} which makes it possible to follow the movements of proteins along nerve axons back towards the cell body. This retrograde movement is of physiological importance because it allows nerve growth factor (NGF) and possibly other materials released by innervated cells to reach the afferent cell bodies to modulate their metabolism. The retrograde axonal path is also taken by tetanus toxin and the neurotropic rabies and Herpes viruses; in the case of tetanus toxin, uptake by the nerve endings depends upon binding to specific sialogangliosides³. Recently, the exploitation of retrograde transport has provided an important new tool in neuro-anatomical investigations. Horse-radiolabelled peroxidase (HRP) injected into the nerve endings can be followed back to the cell body allowing tracing of neuronal circuitry.

The mechanism of fast anterograde transport (50mm per day) from cell body to nerve ending is an energy-dependent process requiring Ca²⁺ ions, intact microtubules and actin⁴. However, the relationship between the smooth endoplasmic reticulum of the axon and neurotransmitter storage vesicles remains unknown. Retrograde transport is much less well understood but there is evidence, based on sensitivity to colchicine, for the involvement of microtubules. Under the electron microscope transported HRP is seen enclosed in membrane-bounded structures suggesting that the smooth endoplasmic reticulum provides the path for transport. However, in a reconstruction of serial sections of optic nerve⁴, HRP

previously injected into the contralateral optic tectum was never found in recognisable profiles of the reticulum even though, in ten instances, the latter's tortuous course was followed for over 4µm. HRP was found in tubular and oval structures and in multivesicular bodies but none of those displayed any continuity with the endoplasmic reticulum.

Other than following the movement of HRP or of ¹²⁵I-labelled proteins taken up by nerve endings and direct light microscopy, there are few techniques for studying retrograde transport. Ligation techniques are of value for studying anterograde transport but not for retrograde transport. The distal segment of the nerve, in which retrograde movement towards the ligation is observed, begins to degenerate once contact is lost with the cell body and these changes occur first in the region of the ligation.

Fink and Gainer's new technique³ enables axonal proteins to be labelled covalently with a radioactive marker (N-succinimidyl [2,3-³H] propionate) following very localised injections into a nerve trunk. The subsequent retrograde and anterograde movements of the labelled proteins can be observed over extended periods of time. Although concentrating on slow transport (1 to 6mm/day), they also showed that the method should be applicable to the study of rapid transport, particularly in the neuro-hypophyseal tract. Whilst the proteins transported

1. Harrison, W. *Nature* 230, 287 (1971).
2. Zink, D. D. *The Stones of Atlantis* (Prentice-Hall, 1978).
3. Rebikoff, D. *Explorers Jr.* 57, 122 (1979).
4. Shinn, E. *Sea Frontiers* 24, 130 (1978).
5. McKusick, M. *Explorers Jr.* 58, 40 (1980).
6. McKusick, M. *Antiquity* 53, 121 (1979).

1. Fink, D.J. & Gainer, H. *Science* 208, 303 (1980).
2. Fink, D.J. & Gainer, H. *J. Cell Biol.* 85, 175 (1980).
3. Schwartz, J.H. *Ann. Rev. Neurobiol.* 2, 467 (1979).
4. Levail, J.H., Rapisardi, S. & Sugino, I.K. *Brain Res.* 191, 3 (1980).
5. Somogyi, P., Hodgson, A.J. & Smith, A.D. *Neuroscience* 4, 1805 (1979).
6. Bentivoglio, M., vander Kooy, D. & Kuypers, H.G.J.M. *Brain Res.* 174, 1 (1979).

Peter Banks is Professor of Biochemistry in the University of Sheffield.

slowly in the centrifugal direction included the well-known cytoskeletal group, there was a single major centripetal species with a molecular weight of 68,000 which could be serum albumin or a fragment of the neurofilament protein.

The value of the HRP/retrograde transport technique has been greatly extended by some ingenious and very beautiful experiments performed at Oxford by Somogyi, Hodgson and Smith⁵. They combined the tracing of the source of afferent endings by HRP with a modified Golgi stain which enabled them to identify the cell type acquiring HRP by its shape and dendritic arborization. By coupling the HRP injection with kainic acid, which destroys cell bodies with receptors for glutamic acid but not their afferent endings, and looking for degenerating boutons on the dendrites of Golgi stained cells which contain HRP, they were able to look for neural feed-back loops.

When they made an electrolytic lesion in the striatum and injected HRP into the ventromedial nucleus of the thalamus, they found that the distal dendrites of Golgi stained/HRP positive cells in the pars reticularis of the substantia nigra carried degenerating synaptic boutons. They thus

demonstrated that a three neurone circuit runs from the striatum and synapses in the substantia nigra and thalamus.

The capriciousness of the Golgi stain also allows the demonstration of stained interneurons synapsing with HRP positive cell bodies and, after placing appropriate lesions, there is also the possibility of detecting degenerating boutons on the interneurons. This multi-methodological approach which combines light and electron microscopy, retrograde transport, Golgi staining and placement of lesions is potentially capable of answering many complex neuroanatomical questions.

The organization of efferent projections from the substantia nigra has also been investigated with the aid of the double retrograde fluorescent labelling technique by Kuypers and his colleagues in Rotterdam⁶ who obtained evidence that some individual neurones projected to both the thalamus and tectum.

Axonal transport in all its manifestations continues to intrigue and occupy neurobiologists. It is perhaps unfortunate that knowledge concerning its mechanism has not yet matched the explosion of information gained from its exploitation. □

(*Proc. natn. Acad. Sci. U.S.A.* **75**, 3751; 1978) found that when vesicles containing membrane proteins are added to the solution below the monolayer, these vesicles establish an equilibrium with the surface monolayer so that some proteins in the vesicles enter the monolayer. When a bilayer is formed, proteins in the monolayer are then incorporated into the newly formed bilayer. Several methods are available for forming vesicles containing live AcChR molecules.

Two reports of successful reconstitution of the AcChR into bilayers using these techniques have just appeared. Schindler and Quast (*Proc. natn. Acad. Sci. U.S.A.* **77**, 3052; 1980) formed liposomes containing receptor from *Torpedo* electroplax, the richest source of the AcChR. Nelson, Anholt, Lindstrom and Montal (*ibid* p. 3057) isolated and purified *Torpedo* AcChR, which was first reconstituted in soybean lipid vesicles using a technique developed by Epstein and Racker, and then incorporated the protein into lipid bilayers. The presence of functional AcChRs in the bilayer was detected by measuring an increase in the permeability to cations with applied, acetylcholine and by showing that single pores had approximately the same conductance and mean open time as the native AcChR in its natural environment. Further, curare — an agent that is a competitive inhibitor of acetylcholine — blocked the effects of the natural agonist in the reconstituted system.

The next step will be to compare the properties of the reconstituted and native molecules. For example, a considerable change in protein dipole moment accompanies the conformational change at pore opening, and as a result the rates of opening and closing depend on the voltage across the membrane containing the AcChR. The effects of transmembrane voltage, as well as temperature and chemical structure of conformational change inducing ligand, will have to be investigated. Similarly, the alkali metal and alkaline earth cations, as well as a number of small (<0.65 nm diameter) positively charged organic ions, pass through the open pore and the relative permeabilities of these ions will have to be determined for the reconstituted AcChR pore. Finally, the effects of the large number of pharmacological agents which alter the AcChR behavior will have to be investigated. In every case differences in the properties of the native and reconstituted molecules will need to be explained in terms of modifications that have occurred either during reconstitution or as result of the altered environment of the reconstituted molecule. When this is done, the advantages of having the reconstituted protein in an easily modifiable environment can be exploited.

As with other multimeric proteins the recently determined partial sequences for the four types of polypeptide chains

The acetylcholine receptor

from Charles F. Stevens

THE acetylcholine receptor (AcChR) has a number of important firsts to its credit. As well as using the first neurotransmitter discovered, acetylcholine, it is the first protein of its class to have the conductance of a single open channel measured (25 pS) and to have the distribution of its pore 'open' times determined (the pore stays open an average of about 1 ms). The AcChR pore was also the first in a biological membrane to have the currents flowing through it measured directly (the single channel current is typically about 2.5 pA). Now this protein has chalked up another first: it has been reconstituted in an artificial planar lipid bilayer.

The acetylcholine receptor is very important because it is a representative of a large class of membrane proteins responsible for the electrical activity of the nervous system. These proteins respond to a signal, either a change in concentration of a neurotransmitter present at the membrane surface or a voltage change across the membrane containing the protein, by opening pores that permit ions to pass through the membrane; the ion flows then produce electrical signals which cause or modulate nerve impulse activity

or, in the case of AcChR, lead through a chain of events to muscle contraction.

The AcChR is composed of 5 subunits, two smaller ones responsible for binding acetylcholine and three different larger ones whose function has not yet been established. The molecule spans the membrane, protruding about 5 nm from the outer membrane surface and 1.5 nm from the inner surface. Its diameter viewed face-on is about 8.5 nm with a 2.5 nm pit on the outer surface forming a vestibule which narrows to an 0.65 nm ion translocating pore.

A great deal of effort has been devoted to trying to get the isolated molecule to function in an artificial lipid bilayer. Techniques that had been successful in incorporating molecules such as the polypeptide antibiotic gramicidin and some larger but not well characterized molecules like excitability-inducing material into bilayers refused to work for the AcChR. Two technical innovations paved the way for the recent successes. First, Montal and Mueller (*Proc. natn. Acad. Sci. U.S.A.* **69**, 3561; 1972) reported a method of forming bilayers from two separately prepared lipid monolayers: lipid is spread at the interface between solution and air on two sides of a teflon partition that has a small hole in it. When the solution levels on both sides are raised above the hole, a lipid bilayer forms over it. Second, Schindler and Rosenbusch

Charles F. Stevens is Professor of Physiology in the School of Medicine at Yale University, and is currently in residence at Cold Spring Harbor Laboratory, New York.

constituting the AcChR show strong homologies, presumably because they evolved through gene duplication. (Raftery, Hunkapiller, Strader & Hood *Science* 208, 1454; 1980). Many large proteins also have homologies within a peptide chain, again indicating tandem duplications of genetic material. The sequence known for the AcChR amounts to only about 10% of the total, too short to permit the detection of such homologies. However, additional parts of the sequence

should be fairly easily obtained by the same techniques. Furthermore, because an estimated 2% of *Torpedo* electroplaque mRNA codes for AcChR, it should be possible to clone and sequence the genes. The base sequence will certainly be helpful in indicating the evolution of the molecule and will be a very important step toward relating the protein structure to its already well described function. The AcChR, then, has a number of chances to add still more firsts in the near future. □

Ultrasensitive mass spectrometry using accelerators

from K. W. Allen

THE detection of long lived radio isotopes such as ^{14}C by mass spectrometric methods using accelerators has a number of important advantages over more conventional methods based on observation of the emitted radiation. First, the sensitivity may be substantially increased, often by several orders of magnitude, so that measurements may be made using milligram samples in a reasonable length of time; second, through the use of a tandem accelerator in which fast negative ions are stripped within the high voltage terminal prior to the second stage of acceleration, discrimination against unwanted stable isobars (for example, N^{14} when detecting ^{14}C) and elimination of molecular ions (for example, $^{13}\text{CH}^-$ which simulates $^{14}\text{C}^-$) by dissociation in the stripper are achieved; and third, the relatively high final energy of the positive ions enables the well established techniques of nuclear particle identification to be employed to advantage.

Ultrasensitive mass spectrometry using the Berkeley 60" cyclotron was first employed by Alvarez and Cornog in 1939 to show that He^3 rather than ^3H was stable, and later (1975) Alvarez and his associates used similar methods to set a very low limit for the natural occurrence of quarks. The need for more sensitive methods of ^{14}C dating in archaeology provided a strong impetus for new developments and in 1977 it was shown independently (see Müller *Physics Today* Feb. 23; 1979) that both cyclotrons and tandem Van de Graaff generators could be used successfully for ^{14}C dating. Within a year or two of these demonstration experiments, approval had been secured for the construction of 3MV tandem generators at the Universities of Arizona, Oxford and Toronto to be dedicated to ^{14}C dating and ultrasensitive mass spectrometry. The first of these new accelerators should be in operation by the end of 1980, but it is likely to be some time before the high accuracy in the $^{14}\text{C}/^{12}\text{C}$ isotopic ratio (<0.5%) essential for dating

of archaeological samples is achieved on a routine basis.

The importance of accelerator based mass spectrometry increases as the lifetime of the radioisotope studied increases, since the specific activity decreases. Thus it is not surprising that accelerator based methods have already been applied to the detection of the long lived cosmogenic nuclides Be^{10} (1.62×10^6 y), Al^{26} (7.16×10^5 y) and Cl^{36} (3.00×10^5 y). The stable isobar at mass 26, Mg^{26} , does not appear to form negative ions so background is not a problem for Al^{26} . However since both Be^{10} and Cl^{36} have stable isobars, it is necessary to rely on chemical separation and specific ionization measurements to remove the background. At 20 MeV, S^{36} and Cl^{36} ions differ in their specific ionization by about 5%, but at $M=100$, the difference for neighbouring isobars is only $\sim 1\%$. Thus separation becomes increasingly difficult with increasing mass. In a recent article, Elmore *et al.* (*Nature* 286, 138; 1980) have shown how a time of flight technique can be used to separate I^{129} (1.6×10^7 years) from I^{127} . Since there are many long lived radionuclides of astrophysical or cosmological interest with $A > 100$, this development is of considerable interest, not only because of the authors' success in measuring $\text{I}^{129}/\text{I}^{127}$ in meteorites at the 5×10^{-12} level, but also because the experiments exposed the limitations of accelerators designed for nuclear physics research when used for ultra sensitive mass spectrometry of heavy elements.

The half life of I^{129} , although long in absolute terms, is short compared with the age of the solar system (4.55×10^9 years) so that all primordial I^{129} has long ago decayed to its stable daughter, Xe^{129} . There has been much analysis and speculation concerning the role of I^{129} decay in relation to the abundances of the Xenon isotopes in meteorites (see Drozd & Podosek *Earth planet. Sci. Lett.* 31, 15; 1976) and the abundance of Xe^{129} in old tellurium bearing rocks has been used to set a lower limit of 1.6×10^{25} years to the lifetime of nucleon decay in Te^{130} (Evans & Steinberg

Science 197, 989; 1977). Since the decay of a proton or a neutron in Te^{130} leads to I^{129} , there would seem to be advantages in detecting I^{129} rather than its stable isobar Xe^{129} as the primordial background would then be eliminated. It should also be possible by accelerator based negative ion mass spectrometry to obtain greater sensitivity, by a factor $\sim 10^2$ to 10^3 , than can be achieved by conventional means. Since the equilibrium yield of I^{129} from nucleon decay, assuming a half life of 10^{30} years, is at most ~ 1300 I^{129} atoms per kilogram of tellurium, improved sensitivity would be very welcome! It will clearly be necessary to obtain tellurium samples from very deep mines to minimise the I^{129} background from radiative neutron capture in Te^{128} , (the neutrons coming from energetic muon interactions), but the occurrence of tellurium in association with gold and silver, which both encourages mining and provides neutron shielding for the tellurium, is a helpful factor. While the sensitivity of geochemical methods such as that described above cannot approach that obtainable with the very large cerenkov detectors now under construction for those events in which the entire rest energy of the nucleon is liberated within the detector, the technique is independent of nucleon decay mode and could in principle detect events in which several neutrinos are emitted. Such events are most unlikely to be revealed by direct detection methods.

The detection of nucleon decay is probably the most challenging and difficult application for ultrasensitive mass spectrometry. There are, however, many fields where the technique is likely, after further development, to prove useful. The measurement of the terrestrial abundance of long lived radionuclides such as Pu^{244} , which has a bearing on the origin of the solar system (Hoffman *et al.* *Nature* 234, 132; 1971), the detection of solar neutrinos, the search for superheavy elements and the detection of rare stable nuclides at a level of parts per billion are all possible areas of application. The recent work on I^{129} (Elmore *et al.* loc. cit.) emphasizes the value of time of flight techniques and the importance of good resolution at injection to eliminate undesirable negative ions; the heavier the ion, the more important these considerations will be.

It seems likely that, over the next few years, tandem generators which are now too small for effective nuclear physics research will be put to good use as ultrasensitive mass spectrometers. It will be necessary to install high resolution injectors on these accelerators, improve the vacuum systems, and perhaps develop new types of detector, especially for the heavier elements. These improvements are relatively modest however, and it should be possible for small groups working on their own University campuses to make important contributions in many different fields. □

K. W. Allen is Professor of Nuclear Structure at the University of Oxford.

ARTICLES

Bunching of 8–10 keV auroral electrons near an artificial electron beam

M. P. Gough*, B. N. Maehlum†, G. Martelli*, P. N. Smith* & G. Ventura‡

*School of Mathematical and Physical Sciences, University of Sussex, Brighton BN1 9QH, UK

†Norwegian Defence Research Establishment, Kjeller, Norway

‡University of Florence, Florence, Italy

High frequency modulations (~1 MHz) of energetic (keV) electrons in the auroral ionosphere have been detected unambiguously for the first time using a novel technique. These modulations were induced by an artificially injected pulsed electron beam (8 keV, 4% duty cycle), and were accompanied by electrostatic wave emissions at the same frequency.

IF waves are excited by a charged beam in a magnetoactive plasma, the beam particles should become bunched at or near the frequency of the excited waves. This is also true for the auroral ionosphere, where such bunching effects could be a useful diagnostic technique. Hitherto these effects could not be studied because of the limited bandwidth of rocket telemetry systems. A technique which overcomes this problem was applied here to the semi-controlled conditions provided by a rocket borne 'mother-daughter' experiment incorporating an artificially injected electron beam.

Several experiments have been carried out with electron guns mounted on rocket payloads¹⁻³. Emissions created by these electron beams are observed up to frequencies beyond the electron plasma frequency. Cartwright and Kellogg⁴, however, noted that most of the radiated beam power goes into waves propagating below the local electron gyro-frequency f_{ce} (~1.4 MHz) as whistler mode waves.

More recently, similar emissions have been observed in natural auroras by receivers operated on the ground^{5,6}. Kisabeth and Rostoker⁶ further identify the source of the emissions below f_{ce} as field aligned currents with the highest frequencies below f_{ce} associated with electron beams in discrete auroral arcs.

We present here results from an artificial auroral beam experiment in which beam-induced bunching of the energetic auroral electrons is observed at a wave frequency of $0.7 f_{ce}$. The only previous attempt⁷ to detect bunching or modulation of auroral electrons up to megahertz frequencies yielded an ambiguously low level of modulation around the detection threshold of the instrument.

Observational technique

On 27 November 1978 at 18.55.35 UT, a Nike Tomahawk rocket (Electron 2) was launched from the Andoya Rocket Range, 69° 17' N, 16° 01' E, Northern Norway, during natural auroral activity. The main payload or 'mother' was instrumented to measure energetic particles, waves and ambient plasma while a separating sub-payload or 'daughter' contained an electron gun producing at peak current a 100 mA beam of 8-keV electrons. The daughter separated at 70 s (95-km altitude) with a relative forward velocity of 0.5 m before the gun pulse sequence started at 92 s and useful particle measurements were made on the mother between 80 s (108 km on the upleg) and 351 s (108 km on the downleg).

On the mother one of the electrostatic analysers was operated at fixed energy 8–10 keV with near omnidirectional opening angle of $190 \times 5^\circ$, to monitor both the background and the gun electrons. The channeltron pulses from this detector were analysed as described below to investigate bunching up to frequencies of 5 MHz. Electrostatic and electromagnetic waves measured on the mother by a pair of boom-mounted elec-

trostatic sensors connected to a receiver sweeping between 0.2 and 3.8 MHz (every 30 ms with a bandwidth of 100 kHz) were recorded simultaneously.

The megahertz bunching analysis of the detected 8-keV electrons was performed by on-board processing of the channeltron detector pulses described fully by Gough⁸. The time interval between subsequent pulses produced by 8 keV electrons was measured as the number of cycles of a 10 MHz crystal oscillator. If the electrons arrive randomly at an average rate $C \text{ s}^{-1}$, then the probability $P(M)$ of two electrons arriving separated by M cycles of the 10-MHz clock will be given by

$$P(M) = P_0 e^{-MP_0} \quad (1)$$

where

$$P_0 = C/10^7 \ll 1$$

Any bunching of the electrons at frequency F (due, for example, to wave-particle interactions) should appear as a cyclic modulation with periodicity $10^7/F$ cycles superimposed on top of the normal non-bunched distribution given by equation (1).

The most probable measurements are those of the shortest time intervals separating two pulses. Given a sufficient accumulation time this makes the technique sensitive to frequencies orders of magnitude greater than the count-rate, but limited by the sampling theorem to a maximum frequency of one half of the crystal clock frequency.

In this preliminary experiment only the time interval between the last two electrons arriving before each telemetry readout was transmitted to the ground. Thus only 400 measurements were available each second, although the average electron arrival rate was between 5×10^3 and $5 \times 10^4 \text{ s}^{-1}$.

If we call $N(M)$ the number of observed pairs of electrons separated by M clock cycles, any bunching at frequency F will produce in the array $N(M)$ maxima around $M = 0$, $M = 10^7/F$, $M = 2 \times 10^7/F$, and so on. Consequently each data set was searched for the presence of electron bunching at 140 frequency intervals spread linearly between 0.2 and 5 MHz. For each frequency trial, the distribution $N(M)$ was multiplied by a square wave of amplitude ± 1 lasting four cycles with phase zero at $M = 0$ (the zero being corrected for electronics dead time).

In the absence of bunching, the distribution of the entries $N(0)$, $N(1)$, $N(2)$, and so on, are approximately poissonian and independent. The difference between the number of counts accumulated in the positive parts of the square wave over those in the negative parts divided by the square root of the total number of counts encompassed by the square wave is expressed as the ratio Z . A 'non-bunched' distribution would give $Z \sim 1$. It follows that the probability of a non-bunched distribution giving $Z > 3$ is 0.0014, and $Z > 2$ is 0.025, for each frequency trial.

Experimental results

Figure 1 shows eight sequential data sets of 33.5 s (five spins of the rocket) analysed in the way described above, with Z plotted whenever it exceeded +1.0. The scale is linear and the $Z = +3$ level is given by the base line of the previous data set. This type of frequency analysis enables us to determine fundamental bunching frequencies, although spurious higher harmonics may also appear. The most striking feature of Fig. 1 is a peak which moves down from 1.0 MHz in the data set centred on 99 s to 0.8 MHz in the data set centred on 232 s. The fact that the spectral pattern repeats itself in five consecutive data sets is highly significant.

As in this frequency band the maximum values of Z are 3.5, 4.1, 2.5, 4.0 and 3.4 for the first five spectra respectively, the electrons can be said to have been definitely bunched in the 0.8–1.0 MHz range.

The electrostatic wave sensors on the mother payload were designed to measure waves in this frequency range. Wave bursts associated with the gun pulses were observed in the same narrow

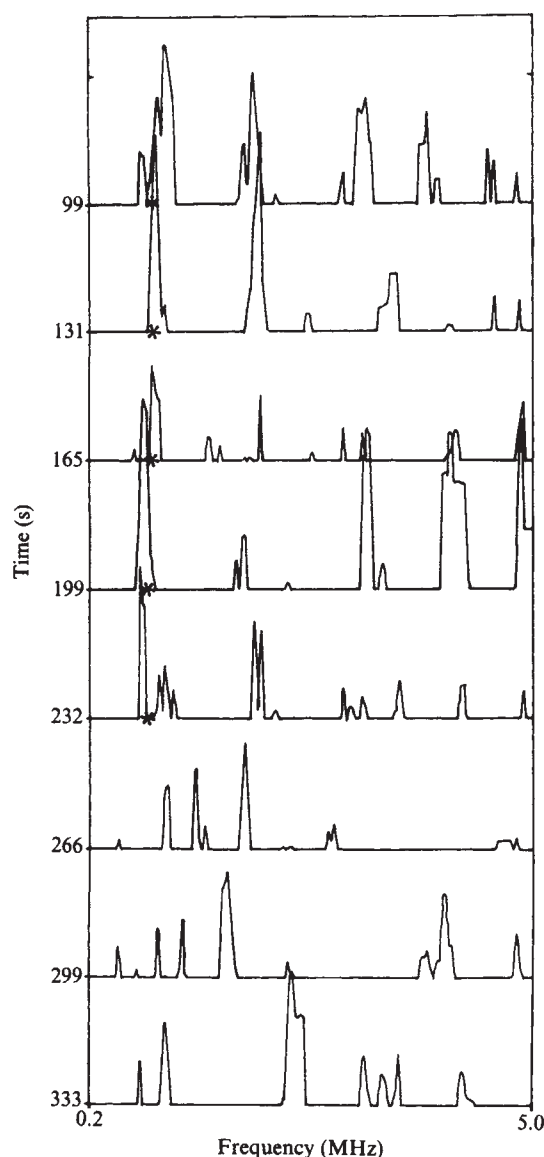


Fig. 1 The observed bunching frequencies of the 8-keV electrons. 33.5 s (five spins) groups of data are presented with Z (see text) plotted against frequency when above $Z = +1$ (baseline) where $Z = +3$ is the baseline of the previous data set. *, Position of the emission observed on the electric field booms. The frequency scale is linear from 200 kHz to 5 MHz.

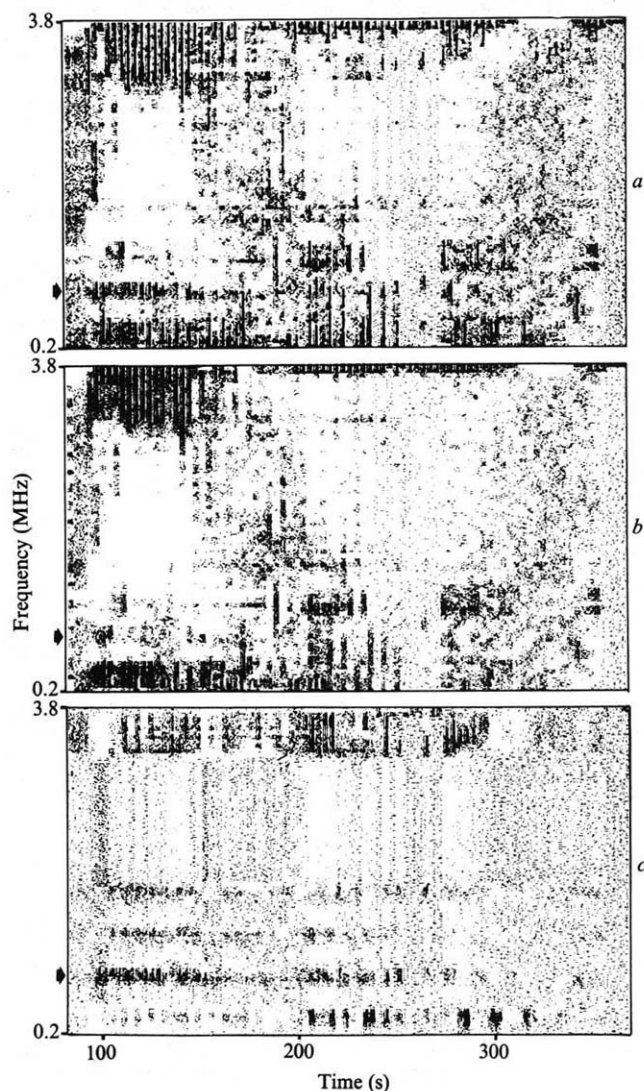


Fig. 2 3.4 s average spectra of electrostatic emissions observed by the electric boom-mounted sensors after subtraction of the average spectrum from 300 to 350 s to remove local interference signals. *a*, All data; *b*, data in between gun pulses; *c*, data during gun pulses. The arrow indicates the position of the emission at $0.7 f_{ce}$. The frequency scales are linear from 200 kHz to 3.8 MHz.

frequency band. Unfortunately the booms on which the sensors were mounted did not deploy fully and the data appear to be contaminated by interference, possibly generated within the payload.

We can assume that towards the end of the flight, the effect of the gun electrons would be much reduced since the mother and daughter reach by then maximum separation, while the interference, if generated in the payload, should not change much. This allows us to correct for interference effects. Figure 2 shows grey scaled 3.4-s average spectra ($\frac{1}{2}$ spin) displayed after subtracting from them the average spectrum for the last part of the rocket flight (300–350 s).

Figure 2a shows all the wave data, Fig. 2b the wave data recorded in between gun pulses, and Fig. 2c the wave data during gun pulses. A distinctive feature of Fig. 2a is an emission in the band 0.8–1.0 MHz that persists from 95 s until ~260 s where it disappears in the noise. This emission is much reduced in Fig. 2b but enhanced in Fig. 2c to be the most distinctive feature in the spectrum. Clearly the emission is directly gun associated and decays rapidly at the end of a gun pulse. The average position of its peak during the 33.5-s data sets of the particle bunching is plotted on the base lines of Fig. 1 as a star for

comparison. The close fit clearly shows that the frequency of the electron bunching coincides with that of the observed electrostatic wave. There is no conceivable way in which the two instruments could interfere with each other and we conclude the observed bunching effects are real.

Figure 2 shows clearly that the emission begins soon after 92 s, when the electron gun is switched on. A study of shorter 6.7-s (1 spin) data sets of the electron bunching indicates that no bunching occurred before this time. Similar data sets show that the electron bunching ceases between 250 and 260 s. At this time, the separation between the mother and daughter is ~ 40 m perpendicular to the geomagnetic field lines. A previous experiment carrying a similar electron gun⁹ showed that at this separation the contribution to the observed fluxes by the gun electrons was vanishingly small.

The electron gun was pulsed every 163 ms with a pulse pattern lasting no more than 9.6 ms. The pulse pattern alternated between a single pulse of 9.6 ms, a single pulse of 0.96 ms or a group of six pulses each 1.9 ms. The electron bunching measurements were consequently sorted into three separate data sets for the period 80–260 s into the flight.

The first set contained all measurements made within 54 ms time bins centred on all electron gun pulses (of all three types), the second set corresponds to the next 54-ms time bins and the third set corresponds to the last 54-ms time bins before those containing the next gun pulse pattern. The values of Z of the peak observed around the 1-MHz frequency interval were 3.6, 2.9 and 1.9 respectively, thus showing that the bunching is directly related to the electron gun pulses. However, the measurements are carried out evenly in time but the duty cycle of the gun is much less than unity. Most of the recorded electrons are, therefore, of auroral origin with the observed bunching induced in them by the electron gun pulses.

Discussion

Typical electron plasma frequencies at this altitude are many megahertz, and we would expect to observe electron bunching around the electron plasma and upper hybrid frequencies. However, electron bunching and wave emission occurred at 0.8–1.0 MHz, that is around 0.7 of the local electron gyro-frequency f_{ce} . Several apparently significant peaks ($Z > 2$) occur above 1 MHz in the bunching spectra but, as mentioned above,

we have ignored them as they may be spurious artefacts of the method of frequency analysis used here. We have, therefore, limited our discussion to the 0.9-MHz signal.

Jones and Kellogg¹⁰ have provided the theory most relevant to the case of an artificial electron beam in the auroral ionosphere. Using beam-plasma parameters different from those in our case, they have shown that waves will grow in the electrostatic whistler mode but with a small growth rate. Results of calculations using parameters closer to those expected in our experiment show that the electrostatic whistler mode waves (or obliquely propagating electron plasma waves) are unstable in the region of $0.7 f_{ce}$ (V. K. Jain, personal communication). Maggs^{11,12} has shown theoretically that natural electrostatic whistler noise undergoes convective beam amplification if there is a positive slope in the velocity distribution. Such a slope has been seen in natural auroral electron beams associated with waves of this type⁶. As noted above, the measured bunching occurred mainly in the natural auroral electrons, and this effect may have been enhanced or induced by the presence of the beam.

Bunching of naturally occurring intense auroral beams can also be expected, and this technique promises to be a valuable diagnostic tool in advancing our understanding of the processes involved in the aurora.

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1. Winckler, J. R., Arnoldy, R. L. & Hendrickson, R. A. *J. geophys. Res.* **80**, 2083 (1975).
2. Monson, S. J., Kellogg, P. J. & Cartwright, D. G. *J. geophys. Res.* **81**, 2193 (1976).
3. Monson, S. J. & Kellogg, P. J. *Cosmic Phys. Tech. Rep.* 172 (University of Minnesota, 1977).
4. Cartwright, D. G. & Kellogg, P. J. *J. geophys. Res.* **79**, 1439 (1974).
5. Kellogg, P. J. & Monson, S. J. *Geophys. Res. Lett.* **6**, 297 (1979).
6. Kisabeth, J. L. & Rostoker, G. *J. geophys. Res.* **84**, 853 (1979).
7. Spiger, R. J., Murphree, J. S., Anderson, H. R. & Loewenstein, R. F. *J. geophys. Res.* **81**, 1269 (1976).
8. Gough, M. P. *Nucl. Instrum. Meth.* (in the press).
9. Maehlum, B. N., Grandal, B., Jacobsen, T. A. & Troeim, J. *Planet. Space Sci.* (in the press).
10. Jones, T. W. & Kellogg, P. J. *J. geophys. Res.* **78**, 2166 (1973).
11. Maggs, J. E. *J. geophys. Res.* **81**, 1707 (1976).
12. Maggs, J. E. *J. geophys. Res.* **83**, 3173 (1978).

Nicholson's blowflies revisited

W. S. C. Gurney, S. P. Blythe & R. M. Nisbet

Department of Applied Physics, University of Strathclyde, Glasgow G4 0NG, UK

A simple time-delay model of laboratory insect populations which postulates a 'humped' relationship between future adult recruitment and current adult population gives good quantitative agreement with Nicholson's classic blowfly data and explains the appearance of narrow 'discrete' generations in cycling populations.

NUMEROUS elementary ecology texts use as illustrative examples of oscillatory population fluctuations, data drawn from the comprehensive and elegant experiments performed by Nicholson^{1,2} on laboratory cultures of the sheep blowfly *Lucilia cuprina*; in these, the population was regulated by the rate of food supply to either the adult population (Fig. 1a) or the larval population (Fig. 1b, c). Notwithstanding their popularity as alleged examples of oscillatory behaviour, the large, quasi-periodic population fluctuations observed by Nicholson are still very imperfectly understood. It is clear from the work of Maynard Smith³ and May⁴ that a combination of high fertility and long development delay must be primarily responsible, and the discrete-time model developed by Varley, Gradwell and Hassell⁵ further suggests that a degree of 'overcompensation' in

the controlling density dependence may be important. However, beyond these rather generalized insights progress has been slight. Despite a variety of more or less sophisticated attempts⁴⁻⁶, no theoretical model has yet yielded a truly satisfactory quantitative fit to the time history of even a single culture, still less has it been possible to formulate a comprehensive framework within which the various subtly different experimental results can be systematically inter-related.

In this article, we seek to formulate a model capable of providing such a framework, but as an initially less grandiose target we shall try to answer a number of specific questions about Nicholson's blowfly data. First, we must identify the class of mechanism which produces the quasi-periodic fluctuations; are they self-sustaining limit cycles perturbed by experimental

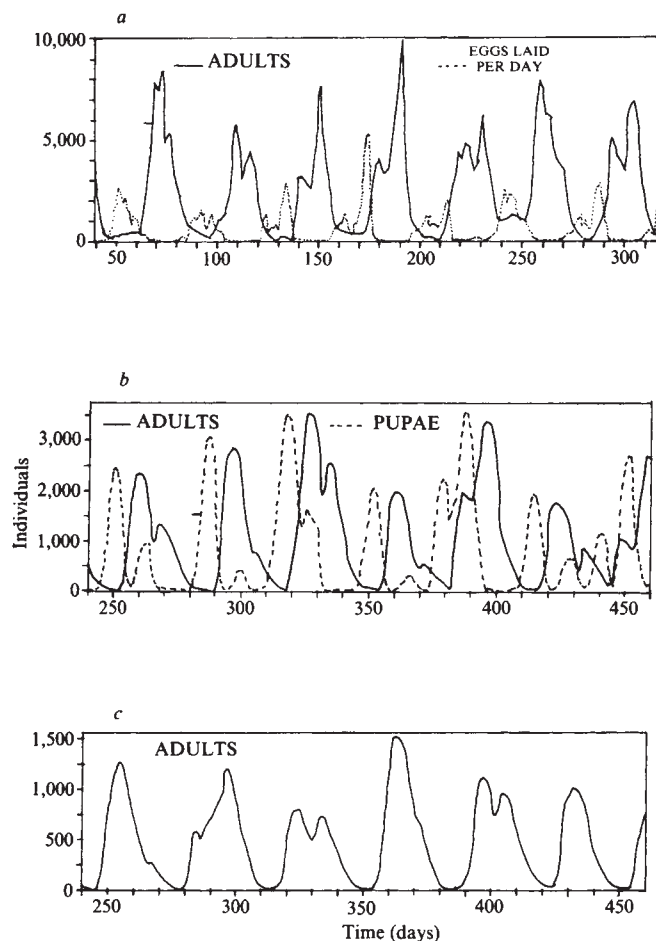


Fig. 1 Quasi-periodic fluctuations in laboratory populations of *Lucilia cuprina*. Population regulated by adult food supply (a), larval food supply (50 g per day) (b), larval food supply (25 g per day) (c). Data from ref. 1.

uncertainty and demographic stochasticity, or are they true quasi-cycles⁷ driven by demographic or environmental stochasticity? Second, we wish to know what determines the period and amplitude of the cycles. Lastly and perhaps most interestingly, we wish to understand the observed patterns of breeding activity; specifically, why the two discrete generations per cycle observed in the larval food-limited case (Fig. 1b) occur at uneven time intervals, and why in the adult food-limited case (Fig. 1a) they merge into a single, almost continuous period of reproductive activity.

The time-delayed logistic model

If it is to be capable of answering questions about the form and timing of generations, our model clearly cannot simply assume that generations are discrete; rather, it must make the initial assumption of overlapping generations and its dynamics must be such as to cause the spontaneous appearance of clearly marked discrete generations in the appropriate conditions. This implies that it must be formulated in terms of a differential, rather than a difference, equation. Probably the simplest single-species continuous time model capable of generating cyclic or quasi-cyclic fluctuations is the time-delayed logistic

$$\frac{dN(t)}{dt} = rN \left[1 - \frac{N(t - T_D)}{K} \right] \quad (1)$$

May⁴ showed that this model gave an acceptable gross fit to the adult population data shown in Fig. 1a with a delay time T_D of about 9 days, a value which he argued compared acceptably with the observed delay time. However, there are several serious

objections to the time-delayed logistic model both in general and as a specific explanation of Nicholson's data. First, close examination of the life history data² shows that the appropriate experimental value of T_D is 14.8 ± 0.4 days, which implies that the discrepancy between 'best fit' and experimental values is worryingly large compared with the confidence interval in the best fit value (± 0.5 days). Second, the model "mixes up time-lagged and not time-lagged contributions" (R. M. May, personal communication) in a way which makes it a very poor paradigm for biological phenomena. However, even more serious than either of these objections is the fact that the time-delayed logistic model is entirely incapable of predicting two bursts of reproductive activity per adult population cycle. It is thus structurally incapable of explaining the results obtained by Nicholson in the larval food-limited regime.

A more realistic model

We now formulate an improved continuous time model of an insect population growing in an isolated laboratory culture. As the experimental data which will serve as the primary quantitative test of the model are mainly based on observations of adult population as a function of time, we begin by writing down a balance equation for the population of sexually mature adults, $N(t)$. The experimental conditions clearly preclude any immigration or emigration and thus the rate of change of N must simply be the difference between the total adult death rate (D) and the rate of recruitment to the adult population (R). If we assume that the *per capita* adult death rate has a time- and density-independent value δ , then

$$\frac{dN}{dt} = R - D = R - \delta N \quad (2)$$

The adult recruitment rate, R , should be evaluated by writing down analogous balance equations for all the life history stages of the species concerned. However, we can evade the resulting complexity (and increase the generality of our model) with the aid of three simplifying assumptions: (1) the rate at which eggs are produced depends only on the current size of the adult population; (2) all eggs which develop into sexually mature adults take exactly T_D time units to do so; (3) the probability of a given egg maturing into a viable adult depends only on the number of competitors of the same age. Together, these assumptions imply that the rate of recruitment at time t can only be a function of the size of the adult population at time $t - T_D$

$$R = R(N(t - T_D)) \quad (3)$$

so that the entire population dynamic can be expressed by a single delay-differential equation

$$\frac{dN}{dt} = R(N(t - T_D)) - \delta N(t) \quad (4)$$

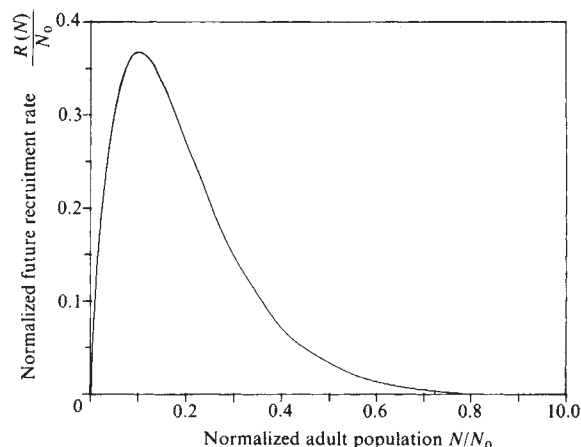


Fig. 2 Assumed dependence of future recruitment rate against present adult population.

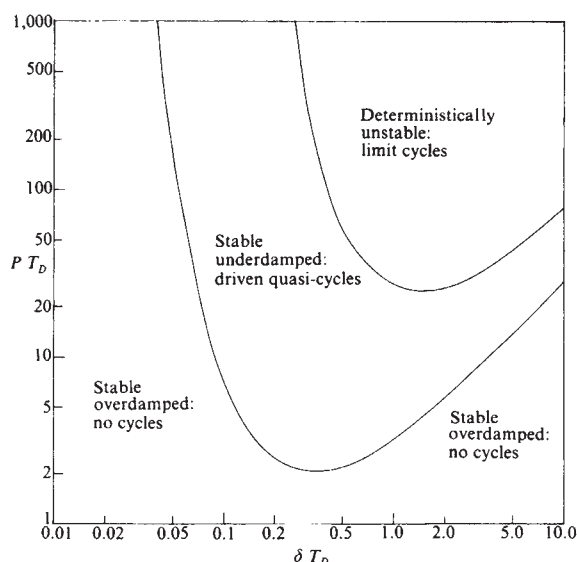


Fig. 3 Qualitative behaviour of the model as a function of the controlling parameters PT_D and δT_D .

Although the recruitment rate function $R(N)$ can in principle be subject to experimental measurement, we wish our model to act as a paradigm for a rather larger class of similar models as well as provide a detailed fit to Nicholson's data, and we therefore seek rather to write down an appropriate algebraic form for $R(N)$. We consider first those experiments in which the only limiting factor is the rate at which food is supplied to the adult population. Here, egg to adult survival may reasonably be expected to be density independent, so that the rate of adult recruitment at time t will be directly proportional to the rate at which eggs were being laid at time $t - T_D$. Provided that the population sex ratio remains reasonably constant, it seems plausible to suppose that, in the presence of excess food, the total rate at which eggs are produced by a population of N adults will be directly proportional to N . However, when food is supplied at a limited rate, intraspecific competition will clearly act to reduce the average *per capita* fecundity of the members of large populations to well below its physiological saturation value. Indeed, where competition is of the 'scramble' type¹, it seems highly likely that very large populations will require the whole of their restricted food intake for physiological maintenance and will thus produce no offspring at all. Clear experimental evidence of such an effect can be found in Fig. 1a, which shows that in Nicholson's adult food-limited culture the total egg production rate drops to zero at high populations. Any plausible functional form for $R(N)$ must therefore go to zero as N becomes either very large or very small. In addition, it seems likely that most recruitment curves will display a single maximum (see Fig. 2) at an intermediate population whose size is determined by the available resources. We therefore choose to represent $R(N)$ by a simple function which displays all these properties

$$R(N) = PN \exp \{-N/N_0\} \quad (5)$$

where P is the maximum possible *per capita* egg production rate (corrected for egg to adult survival) and N_0 is the population size at which the population as a whole achieves maximum reproductive success.

In experiments in which the controlling factor is the rate of food supply to the larval population, the situation is at first sight entirely different. Here, the adults are always provided with excess food and thus always produce eggs at their physiological maximum rate. However, competition among the larvae now makes egg to adult survival highly density dependent, and indeed, Nicholson's batch culture experiments strongly suggest that when an age class is very large, none of its members will

actually pupate successfully. Thus, within the limitations of our assumption that egg to adult survival is affected only by competition within a given age class, we can use equation (5) to describe recruitment in both the adult food-limited and the larval food-limited cases.

The dynamics of our model are thus always described by

$$\frac{dN(t)}{dt} = PN(t - T_D) \exp \{-N(t - T_D)/N_0\} - \delta N(t) \quad (6)$$

which has a single non-trivial stationary state

$$N^* = N_D \ln(P/\delta) \quad (7)$$

It can easily be shown that the local stability of the steady state and the qualitative properties of the fluctuations about it are entirely controlled by the two quantities PT_D and δT_D , and in Fig. 3 we show the disposition in the $PT_D/\delta T_D$ plane of the various regions of qualitatively different behaviour. Because we seek to understand fluctuations which are at least quasi-periodic, we shall restrict our detailed analysis to the stable/underdamped (damped oscillatory) and locally unstable regions.

If the parameters of our deterministic model are in the stable/underdamped region, we would expect the demographic stochasticity present in a real system to induce quasi-cyclic population fluctuations consisting of bursts of relatively coherent cycles whose period is close to the deterministic natural period, interspersed with short periods of incoherent noise⁷. If we describe the return of an underdamped system to its deterministic equilibrium by a coherence number n_c , defined as the number of cycles over which the amplitude of the transient is reduced by a factor e , then it is found empirically⁸ that when such a system is executing driven quasi-cycles, each burst of coherent cycles contains an average of roughly $3n_c$ cycles. Thus, in Fig. 4 we characterize the quasi-cyclic behaviour of our model in the stable/underdamped regime by plotting contours of constant normalized cycle period (T/T_D) and constant coherence number n_c on the appropriate part of the $PT_D/\delta T_D$ plane.

For model parameters in the locally unstable region, the exact solution of equation (6), which can be obtained by numerical integration, takes the form of a self-sustaining oscillation. For some parameter values this is a simple limit cycle, whereas for

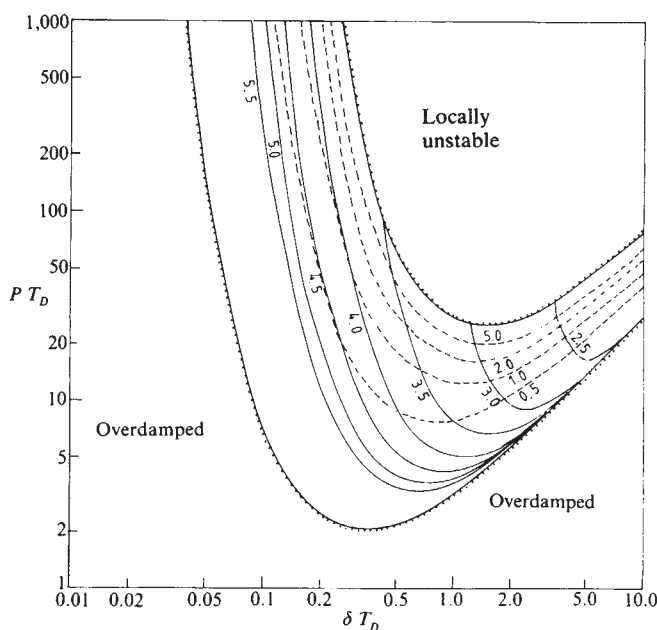


Fig. 4 Characterization of quasi-cyclic behaviour in the stable/underdamped region. Contours of constant normalized period T/T_D (—) and coherence number (---) in the $PT_D/\delta T_D$ plane.

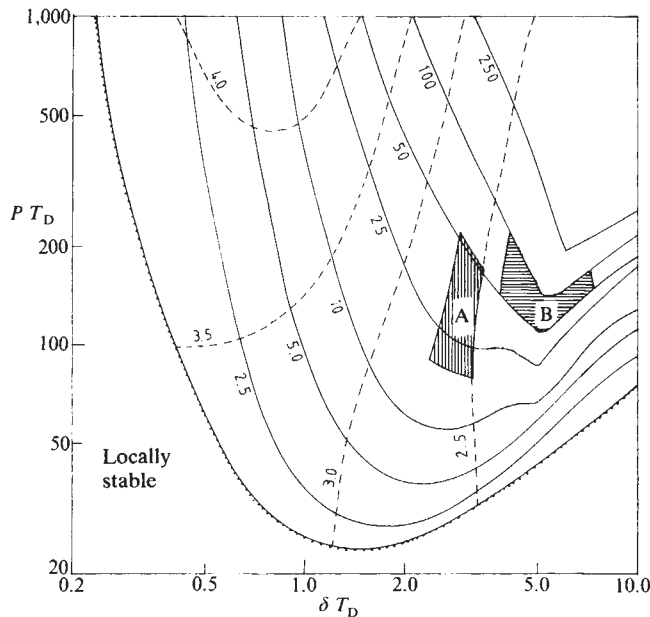


Fig. 5 Characterization of limit cycle behaviour in the locally unstable region. Contour of constant normalized period T/T_D (---) and population ratio $N_{\max}/N_{\min}$ (—) in the $PT_D/\delta T_D$ plane. The area marked A shows the best fit parameters for the data of Fig. 1a and the area marked B corresponds to the data of Fig. 1b.

others it is a more complex cycle or is even formally aperiodic. In all cases, however, a spectral analysis of the solution reveals a single clearly marked dominant period (T). We have therefore characterized the solutions in this region by the ratio of this dominant period to the development delay (T/T_D) and the ratio of maximum to minimum population observed during the cycle ($N_{\max}/N_{\min}$). In Fig. 5 we plot contours of these quantities in the appropriate part of the $PT_D/\delta T_D$ plane.

An experimental test

As a quantitative test of our model, we now determine the parameter values which give the best fit to the gross characteristics (T/T_D and $N_{\max}/N_{\min}$ or n_c) of the fluctuations in the adult population of Nicholson's blowfly cultures, and then compare these best fit values with independent experimental estimates of the same quantities. We restrict our attention to the adult food-limited case (Fig. 1a), because only here do the data allow us to make an accurate independent estimate of the maximum *per capita* fecundity (P).

There is no simple test which will tell us *a priori* whether the fluctuations shown in Fig. 1a are self-sustaining limit cycles or driven quasi-cycles, so we must determine the best fit parameters under both hypotheses. If we assume that the fluctuations are of limit cycle type, then our quantitative characterization requires us to measure the maximum to minimum population ratio $N_{\max}/N_{\min}$ and the normalized cycle period T/T_D ; quasi-cyclic fluctuations we characterize by T/T_D and the coherence number n_c . Examination of Fig. 1a combined with our previous identification of the correct value of the delay T_D as 14.8 ± 0.4 days reveals that these quantities lie in the ranges

$$2.5 < (T/T_D) < 2.7; \quad 29 < (N_{\max}/N_{\min}) < 53; \quad 2 < n_c < 5 \quad (8)$$

which enables us to infer from the contour maps given in Figs 4 and 5 that the best fit values of the controlling parameters PT_D and δT_D for the limit-cycle hypothesis are

$$PT_D = 150 \pm 70 \quad \delta T_D = 2.9 \pm 0.5 \quad (9a)$$

and for the quasi-cycle hypothesis

$$PT_D = 23.5 \pm 4.5 \quad \delta T_D = 3.0 \pm 0.7 \quad (9b)$$

Although the best fit values of δT_D required under the two hypotheses are essentially indistinguishable, it is clear that the appropriate values of PT_D differ by more than a factor of 6. Thus, an independent estimate of the maximum *per capita* fecundity P will provide a clear *a posteriori* test of the mechanism underlying the observed cycles. We can easily obtain such an estimate from the egg-laying rate data in Fig. 1a by recognizing that, because the adult population minima are clearly below the maximum of the total reproduction curve, the value of P is simply given by

$$Pe^{-1} = \frac{\text{Maximum rate of egg-laying}}{\text{population producing maximum reproduction}} \quad (10)$$

The value of P thus inferred from Fig. 1a is in the range 7.4 per day $< P < 11.4$ per day, so that our final independent estimate of the controlling parameter PT_D is

$$PT_D = 130 \pm 30 \quad (11)$$

which is clearly compatible with the value required under the limit cycle hypothesis and absolutely incompatible with the quasi-cycle hypothesis. This, together with the fact that an independent estimate of δT_D is in good general agreement with the values required under both hypotheses, gives us considerable confidence that the fluctuations observed by Nicholson in the adult food-limited case are of limit-cycle type.

Separate generations in cycling populations

Our rather simplistic initial characterization of the non-linear behaviour of equation (6) in the limit-cycle region in fact conceals a wealth of complexity, for the formal repeat period of the cycles can often be many times the empirically observed dominant period. The general pattern is very similar to that seen by Mackay and Glass⁹ and May¹⁰ in equations of similar structure; close to the local stability boundary the solution is a simple limit-cycle with period T , but moving deeper into instability produces a number of successive doublings of the repeat time until a region is reached where the solution is formally aperiodic (chaos). Beyond the chaotic region we again see cyclic fluctuations.

These variations in the fine structure of the adult population fluctuations, although mathematically fascinating, are likely to be hard to identify experimentally in the presence of realistic levels of experimental uncertainty. However, the accompanying variations in the predicted pattern of reproductive activity are not only sufficiently dramatic to be easily identified experimentally, but also provide a clear explanation of Nicholson's observation that, in certain experimental circumstances, breeding occurred in quasi-discrete 'generations' but that in others it was much more nearly continuous. The mechanism by which separation of generations can occur in our model is illustrated in Fig. 6. When the minimum adult population in the trough of the cycle ($N_{\min}$) is greater than the population size at which maximum total reproduction occurs, N_0 , then breeding activity ($R(N)$) is essentially continuous with one broad peak per cycle (Fig. 6a). When $N_{\min}$ is slightly less than N_0 (Fig. 6b), the pattern is still much the same but the reproduction rate peak now has a distinctive 'double-humped' shape. However, when $N_{\min} \ll N_0$, the pattern changes dramatically. In simple cases such as Fig. 6c, each adult population peak generates two narrow discrete generations, whereas in more complex cases such as Fig. 6d, when the adult population variation is only quasi-periodic, the generations are also very variable in size.

We have tested the plausibility of this mechanism as an explanation of Nicholson's observations by comparing the

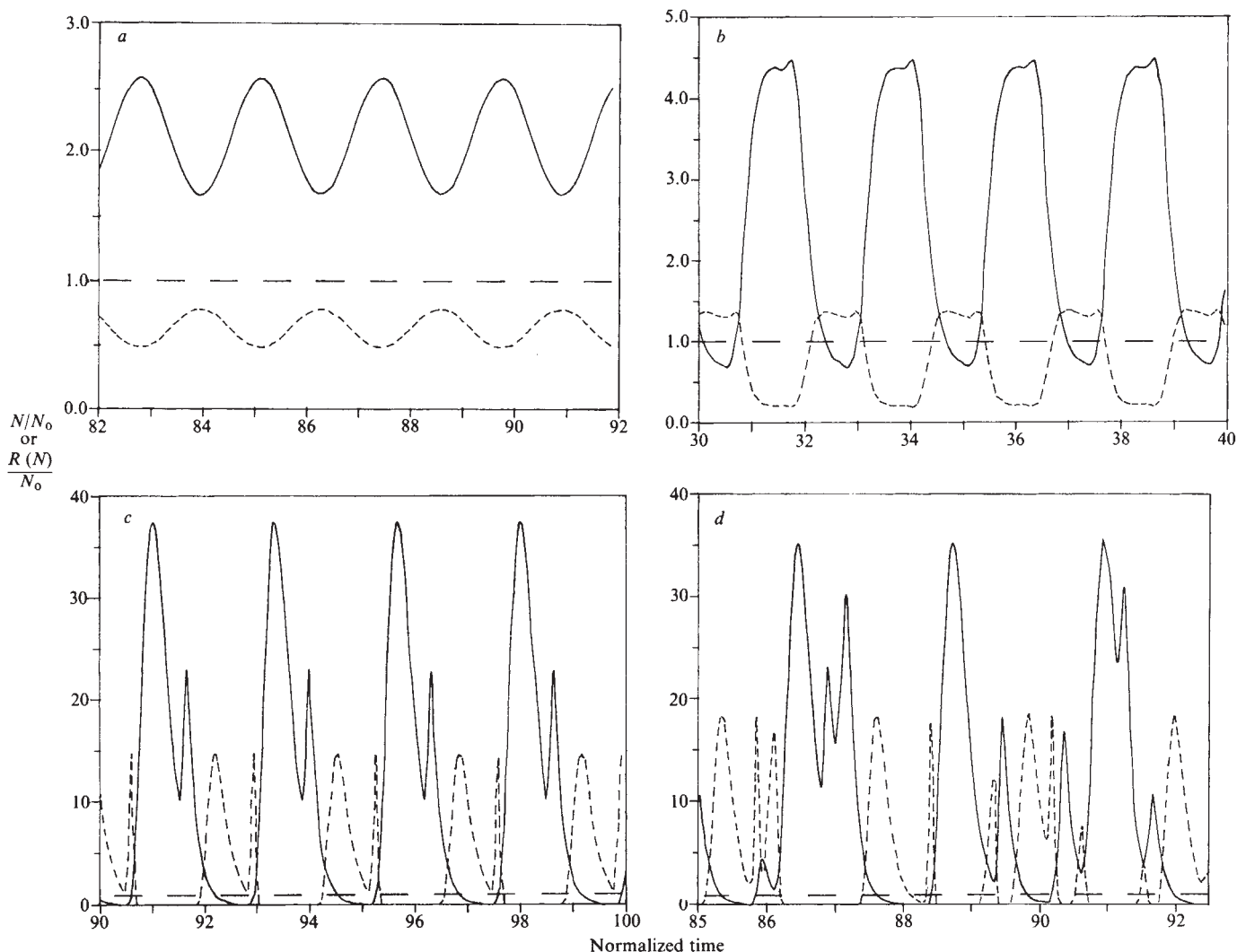


Fig. 6 Separation of generations in a cycling population. Normalized adult population (—) and normalized future recruitment rate (---) as functions of time. *a*, $N_{\min} \gg N_0$: continuous reproduction; *b*, $N_{\min} \sim N_0$: one generation per cycle; *c*, $N_{\min} \ll N_0$: two generations per cycle; *d*, $N_{\min} \ll N_0$ and adult fluctuations aperiodic: complex generation pattern.

patterns of breeding activity predicted by our model with best fit parameters determined from the adult population fluctuations shown in Fig. 1*a* and *b*. For the adult food-limited case (Fig. 1*a*), we predict a single double-humped peak per population cycle, whereas in the larval food-limited case (Fig. 1*b*), we predict that breeding should take place in clearly distinguishable generations occurring at uneven time intervals. It is clear from the egg-laying rate and pupal population data given in Fig. 1*a* and *b*, respectively, that this strongly marked qualitative change is indeed observed experimentally.

Conclusions

The model described above provides a satisfying qualitative fit to Nicholson's blowfly data, and its extreme simplicity thus allows us to deduce that the observed fluctuations arise from the combination of a long development delay with a single-humped total reproduction curve. Moreover, it seems clear that any model containing these two generic features must behave in a generally similar way, so that our picture of the way in which discrete generations may be spontaneously maintained in cycling populations should in fact be rather generally applicable.

This model may thus be the prototype of a family of models which will unify the hitherto diverse 'discrete generation' and

'overlapping generation' views of insect population dynamics and lead to a clearer understanding of the uneven timing of insect generations both in the constant environment of the laboratory and in the variable environments encountered in the field. However, considerable further development will be required before we can fully assess the potential of the framework provided by such models to elucidate the subtle systematic differences in behaviour between various populations. As the range of technically feasible modifications is immense, we strongly believe that further development can only be done efficiently in conjunction with a comprehensive experimental programme.

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1. Nicholson, A. J. *Aust. J. Zool.* **2**, 9–65 (1954).
2. Nicholson, A. J. *Cold Spring Harb. Symp. quant. Biol.* **22**, 153–173 (1957).
3. Maynard Smith, J. *Models in Ecology* (Cambridge University Press, 1974).
4. May, R. M. *Stability and Complexity in Model Ecosystems* (Princeton University Press, 1973).
5. Varley, G. C., Gradwell, G. R. & Hassell, M. P. *Insect Population Ecology* (Blackwell, Oxford, 1973).
6. Auslander, D. M., Oster, G. F. & Huffaker, C. B. *J. Franklin Inst.* **297**, 345–376 (1974).
7. Nisbet, R. M. & Gurney, W. S. C. *Nature* **263**, 319–320 (1976).
8. Nisbet, R. M., Gurney, W. S. C. & Pettipiece, M. *J. theor. Biol.* **68**, 143–160 (1977).
9. Mackay, M. C. & Glass, L. *Science* **197**, 287–289 (1977).
10. May, R. M. *Proc. Int. Conf. on Nonlinear Dynamics* (New York, 1979).

Enkephalin blocks inhibitory pathways in the vertebrate CNS

R. A. Nicoll, B. E. Alger & C. E. Jahr

Departments of Pharmacology and Physiology, University of California, San Francisco, California 94143

Enkephalin markedly attenuates a variety of GABAergic inhibitory pathways in the CNS, but does not affect the action of GABA. The action of enkephalin is reversed by the specific opiate antagonist, naloxone. Thus, inhibitory interneurons may be primary targets for opioid peptide-containing pathways and disinhibition may be of general importance for opioid peptide action in the CNS.

OPIATE receptors and opioid peptides have been found in a variety of central nervous system (CNS) structures¹. However, the distribution of these receptors and peptides gives little indication of their possible physiological role. The cellular action of opiates and opioid peptides in the CNS has been the subject of a number of reports (for review see ref. 2). There is considerable evidence of a presynaptic inhibitory role for opioid peptides³⁻⁶ as well as a direct postsynaptic inhibitory action^{2,7}. In the CNS, iontophoretic application typically inhibits the firing of sensitive neurones. However, a survey of opioid effects on neuronal activity in several brain regions of the rat revealed naloxone-reversible excitatory effects, and in the hippocampus 90% of the pyramidal cells encountered were strongly excited by enkephalin⁷. Further *in vivo* iontophoretic studies by Zieglgänsberger *et al.*⁸ suggested that this excitation may be mediated indirectly by inhibition of neighbouring inhibitory interneurons, resulting in a disinhibition of pyramidal cells. Several recent studies, using primarily extracellular field potential and unit recording in slice preparations, support the proposal that enkephalin depresses the inhibition of pyramidal cells⁹⁻¹⁵. However, a number of questions remain. For instance, different studies have suggested preferential opiate effects on recurrent, or alternatively, on feedforward inhibition, and the site of the opiate effects, whether pre- or postsynaptic, is unknown¹¹. Direct effects on excitation have not been ruled out, and there has been some question about the naloxone reversibility of the opiate action¹⁶. To some extent these difficulties can be attributed to the use of field potential and extracellular unit recording techniques, the results of which may be difficult to interpret. Here, we have used the hippocampal slice preparation and intracellular recording to analyse the cellular actions of enkephalin. In addition, we have extended these results to the *in vitro* olfactory bulb and spinal cord.

Enkephalin blocks i.p.s.ps in hippocampal pyramidal cells

The rat hippocampal slice preparation used in our experiments has been described elsewhere¹⁷. Stimulating electrodes were placed on the alveus for antidromic activation and in the stratum radiatum for orthodromic activation of pyramidal cells. Pentobarbital (0.1 mM) was added to the perfusate in some experiments, not only to enlarge and prolong the inhibitory postsynaptic potentials (i.p.s.ps), but also to unmask a feedforward, dendritic form of inhibition¹⁷. The stable enkephalin analogue, D-Ala²-Met⁵-enkephalinamide (DALA), was used in most experiments and was applied by bath perfusion in known concentrations.

We first tested the effects of DALA on field potentials recorded from the stratum pyramidale in response to orthodromic and antidromic stimulation. In the presence of DALA (5 μ M), the size of the initial pyramidal population spike increases and

additional population spikes appear, although there is no change in the amplitude of the antidromic response (Fig. 1). The facilitation of orthodromic responses is entirely reversed by the addition of naloxone. Concentrations of DALA as low as 10⁻⁸ M were effective, whereas morphine and Met-enkephalin required 10–100-fold higher concentrations. These findings (see refs 9, 10, 12–15) indicate that opiates, by activating opiate receptors, markedly enhance the excitatory synaptic responses of pyramidal cells, while having no demonstrable effect on antidromic responses.

To determine the mechanism for this profound and selective action, we have used intracellular recording from CA1 pyramidal cells. The left-hand column of Fig. 2 illustrates responses to antidromic stimulation and to two intensities of orthodromic stimulation, recorded with a potassium methylsulphate-filled electrode. The substantial reduction in the deflections caused by hyperpolarizing current pulses indicates that there is a fall in membrane resistance during the i.p.s.p. The i.p.s.p. produced by antidromic stimulation is of short duration and thought to be generated by a collateral feedback, interneuronal circuit mediated by γ -aminobutyric acid (GABA)¹⁸. The orthodromic i.p.s.p. evoked by high intensity stimuli has an additional depolarizing component which is considered to be generated by a feedforward pathway involving a set of GABA interneurons distinct from those involved in the feedback pathway¹⁷. DALA has little effect on the membrane potential or resistance of pyramidal cells, and the size of both the orthodromic and antidromic evoked i.p.s.p. is decreased. However, we have consistently found that feedforward inhibitory potentials to orthodromic stimulation are attenuated to a considerably greater extent than the responses to antidromic stimulation

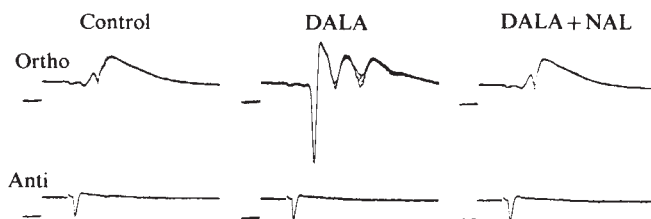


Fig. 1 Effect of opioid peptide (DALA) on field potentials in the hippocampal slice. The left-hand traces show the control responses recorded with an extracellular electrode in the pyramidal cell layer to antidromic (Anti) and orthodromic (Ortho) stimulation. The sharp downward deflections represent the synchronous firing of pyramidal cells and are termed population spikes. The middle traces show that exposure to 5 μ M DALA greatly augments the orthodromic population spike and creates later population spikes while having no effect on the antidromic responses. Addition of naloxone (2 μ M) to the DALA solution (DALA + NAL) completely reverses the effects of DALA. The calibration pulse at the beginning of each trace is 2 mV and 5 ms.

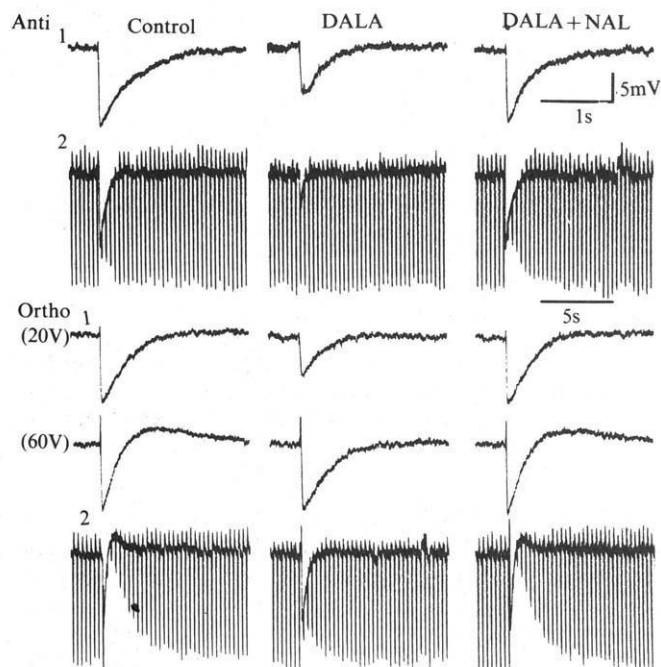


Fig. 2 Opioid peptide (DALA) antagonizes evoked i.p.s.ps in a hippocampal pyramidal cell. The responses to antidromic stimulation are shown at a fast (Anti₁) and a slow (Anti₂) chart speed with constant current hyperpolarizing pulses. The orthodromic responses are shown at a fast chart speed (Ortho₁), and at a low (20 V) and high (60 V) stimulus intensity. Note the late slow depolarization with high intensity stimulation. The response to high stimulus intensity is also shown at a slow chart speed (Ortho₂) with constant current hyperpolarizing pulses. The gain is the same in all records. The left-hand column shows the control responses. The records in the middle column were obtained 12 min after switching to 5 μ M DALA, and the records in the right-hand column were obtained 15 min after adding 2 μ M naloxone to the DALA-containing solution. The bathing medium contained 10⁻⁴ M pentobarbital. The resting membrane potential of this cell was -65 mV.

which are generated exclusively by a feedback pathway. In control conditions, orthodromic stimulation elicits a single action potential in pyramidal cells, whereas in the presence of DALA, multiple spikes are usually generated. In all 24 cells examined, DALA attenuated i.p.s.ps and thereby facilitated the ability of an excitatory postsynaptic potential (e.p.s.p.) to fire the cell. These effects of DALA were entirely reversed by naloxone, which by itself had no effect. The attenuation of the i.p.s.p. by DALA was also seen with KCl-filled electrodes, which reversed the i.p.s.ps into large depolarizing potentials (Fig. 3) and resulted in many spontaneous potentials appearing on the baseline (Fig. 4). These depolarizing spontaneous potentials, which are never seen in CA1 pyramidal cells when electrodes are filled with impermeant anions, are blocked by GABA antagonists, prolonged by pentobarbital and abolished by tetrodotoxin¹⁹. Because true quantal release (release of individual packets of transmitter) is resistant to tetrodotoxin blockade of electrical excitability²⁰, we conclude that these potentials represent small i.p.s.ps generated by the spontaneous discharge of inhibitory interneurons. With KCl-filled electrodes, cells typically hyperpolarized a few millivolts when exposed to DALA; this resulted from a cessation of spontaneous i.p.s.ps ($n = 7$). The film records in Fig. 4 illustrate this blocking action of DALA on the potentials and its reversal by naloxone.

The present findings indicate that the primary effect of DALA on pyramidal cells is indirect and involves a disinhibition, as proposed previously⁸. Although the effects of GABA antagonists are virtually identical to those reported here for DALA (see also refs 9–15), the DALA antagonism does not occur at the level of the GABA receptor, because the responses to exogenous GABA are not antagonized by DALA (Fig. 3).

We consider the blockade of spontaneous i.p.s.ps by DALA to suggest that DALA exerts its effect at the level of the cell body of the inhibitory interneurone, suppressing cell discharge, although a more direct action on GABAergic synaptic terminals^{3–6} cannot be excluded by these results. It is interesting that the opiate receptor distribution in the CA1 region determined autoradiographically²¹ would agree well with the location of interneurons thought to mediate inhibition¹⁸. Immunohistochemical studies of the hippocampus²² suggest that the enkephalin-containing fibres and terminals are present mainly in the very small CA2 field. Perhaps a non-enkephalin, opioid-containing fibre system exists in CA1, or perhaps enkephalin is capable of diffusing from CA2 to the nearby CA1.

Enkephalin blocks dendrodendritic inhibition in the olfactory bulb

We have examined the effects of DALA on other forms of inhibition in the CNS to determine how widespread a phenomenon disinhibition might be. We have recently developed an isolated turtle olfactory bulb preparation to examine dendrodendritic inhibition of mitral cells, using intracellular recording²³. In all cells tested ($n = 7$), DALA markedly attenuated i.p.s.ps evoked either orthodromically or antidromically, and was particularly effective against those generated at reciprocal synapses by direct depolarization of the mitral cell (Fig. 5, middle column). This effect occurred with little change in the resting membrane properties of mitral cells. Moreover, naloxone could reverse the i.p.s.p. depression (Fig. 5, right-hand column). Because the directly evoked i.p.s.p. is generated solely by dendritic reciprocal synapses, DALA seems to be acting directly on the reciprocal synapses. This site of action agrees with neurochemical and anatomical results. Opiate receptor binding is high in the layers of the bulb in which reciprocal synapses exist²⁴. Anatomical studies^{25,26} have shown that centrifugal fibres do not make direct contact with mitral cells, but end exclusively on inhibitory interneurons. Of particular interest is the finding that some of the centrifugal fibres synapse directly onto the gemmules of granule cells, the structures which form the reciprocal synapses^{25,26}. It will be of interest to see if some of these centrifugal fibres contain enkephalin.

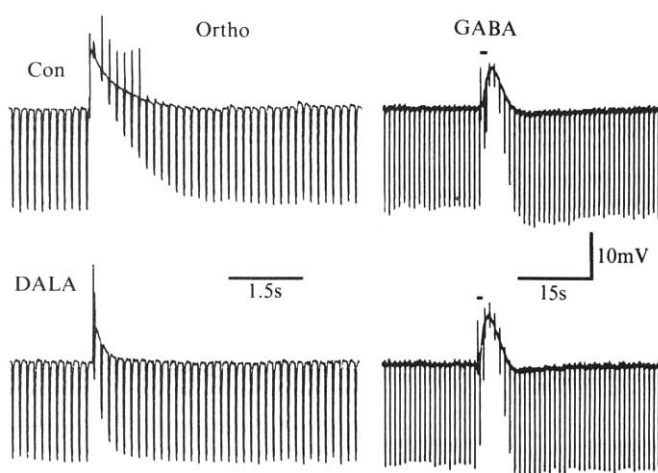


Fig. 3 Opioid peptide (DALA) is not a GABA antagonist. The upper traces show the response to orthodromic stimulation (Ortho) and to an iontophoretic GABA pulse (80 nA) applied to the apical dendrites and recorded from the soma of a pyramidal cell. The lower records were obtained 5 min after switching to a 5 μ M DALA-containing solution. The cell hyperpolarized 3 mV, resulting in a slight increase in the GABA responses. The orthodromic response is markedly curtailed. The bathing medium contained 10⁻⁴ M pentobarbital. The membrane potential was -52 mV. The recording electrode was filled with 3 M KCl.

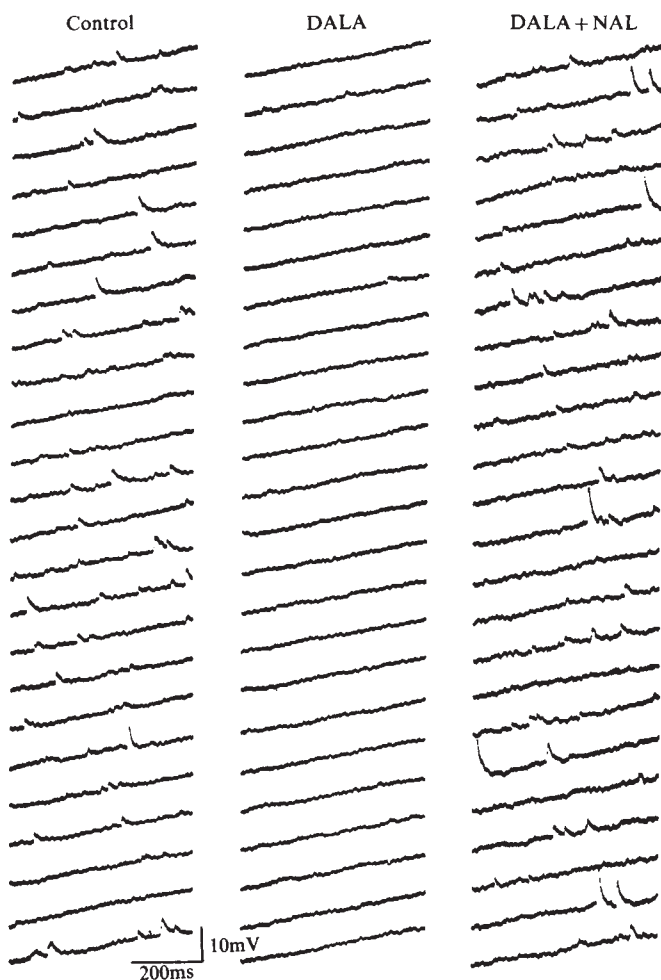


Fig. 4 Opioid peptide (DALA) blocks spontaneous i.p.s.ps in hippocampal pyramidal cell. The responses were recorded on running film with a 3 M-KCl filled microelectrode in the absence of pentobarbital. To block spontaneous action potentials the membrane was hyperpolarized to -69 mV while the film records were obtained. Switching to a solution containing $5 \mu\text{M}$ DALA for 6 min eliminates the spontaneous depolarizing potentials. Addition of $2 \mu\text{M}$ naloxone to the DALA-containing solution reverses the depressant action of DALA. The resting membrane potential was -53 mV in control, -56 mV in DALA and -50 mV in DALA + NAL. The faster decay of the potentials in DALA + NAL was due to a fall in membrane resistance resulting from imperfect sealing of the electrode when these records were obtained.

Enkephalin blocks presynaptic inhibition in the spinal cord

Finally, we have tested the effects of DALA on presynaptic inhibition in the isolated spinal cord of the frog in 20 preparations. The techniques used to stimulate and record from this preparation have been described previously²⁷. In this preparation, stimulation of the ventral root depolarizes primary afferent terminals. This response can be recorded from the dorsal root with extracellular electrodes and is referred to as a dorsal root potential (d.r.p.)^{28,29}. We have found that DALA produces a remarkable and almost complete block of both the d.r.p. (Fig. 6A, B) and the accompanying inhibition of primary afferent transmission (Fig. 6C). This effect is quite selective, for at a time when the d.r.p. is virtually abolished, synaptic excitation by large myelinated primary afferents is either unaffected or actually enhanced. The d.r.p. evoked by stimulating an adjacent dorsal root is less sensitive to DALA. However, both the rising phase of the d.r.p. and the dorsal root reflex (which are considered to be mediated by GABA^{30,31}), are depressed by DALA. Although Met-enkephalin and morphine produced effects similar to DALA in the spinal cord, approximately 100-fold higher concentrations were required. A similar depression of

d.r.ps by enkephalin and morphine has been observed in the isolated rat spinal cord³² and intact cat^{35,36}.

In addition to depressing d.r.ps, DALA hyperpolarizes primary afferents. This effect is due partly to a block of the spontaneously occurring d.r.ps (Fig. 6A) and partly to a direct action on the terminals, as the hyperpolarization is not abolished when synaptic transmission is blocked by magnesium or tetrodotoxin. The block of d.r.ps, presynaptic inhibition and the hyperpolarization are all completely reversed by naloxone (Fig. 6A). GABA, or a closely related neutral amino acid, is thought to be the transmitter responsible for depolarization of primary afferents^{27,29,31}. However, as in the case with hippocampal pyramidal cells, the actions of GABA (Fig. 6B) and β -alanine on primary afferents is unaffected by DALA at a time when the d.r.ps are abolished. Therefore, the action of DALA must occur at some earlier step in this pathway, possibly on cell bodies of presynaptic inhibitory interneurons or directly on the axo-axonic synapses.

Concluding remarks

We have found that excitatory opiate effects in various parts of the vertebrate CNS are caused indirectly, and actually occur as the result of disinhibition. These forms of inhibition include feedforward and recurrent inhibition of hippocampal CA1 pyramidal cells, dendrodendritic postsynaptic inhibition of olfactory bulb mitral cells, and presynaptic inhibition of spinal primary afferents. Because enkephalin does not antagonize the action of GABA, the putative neurotransmitter for these inhibitory pathways, a depression of inhibitory interneuron excitability and/or a block in GABA release from nerve terminals are likely explanations for the disinhibition caused by enkephalin. The depression of putative inhibitory neuronal firing by enkephalin, reported by others, would favour the former explanation^{8,13}.

Such a disinhibitory mechanism at supraspinal sites could help explain the epileptic activity seen following administration of opioid peptides^{33,34}. Although morphine is relatively ineffective in blocking i.p.s.ps, generalized seizures have been reported following high systemic doses³⁷. Furthermore, the present findings suggest that disinhibition may be an important general mechanism of opioid action in the CNS and that local, GABA-containing, inhibitory interneurons are primary targets of enkephalin-containing fibre systems. Such neurones are thought to have a key role in integrating neuronal information within a given region. Very small changes in the release of enkephalin would, by modifying the spontaneous activity of inhibitory

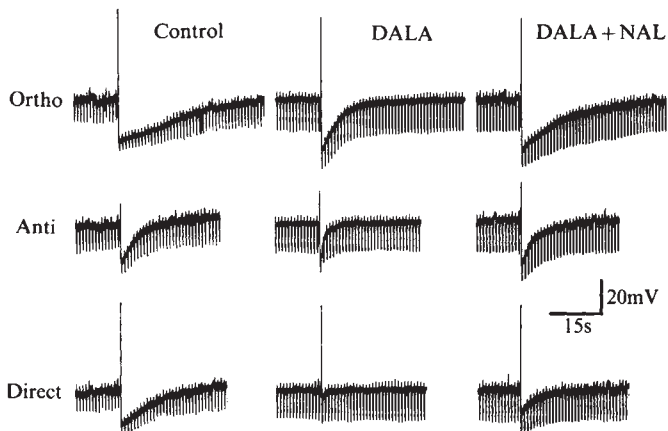


Fig. 5 Opioid peptide (DALA) blocks i.p.s.ps recorded intracellularly from an olfactory bulb mitral cell. The top records were obtained by stimulating the olfactory nerves, the middle records by mitral cell axon stimulation, and the bottom records by direct depolarizing current pulses which discharged the cell. The left-hand column shows the control responses. The middle column shows the responses obtained 20 min after perfusion with a Ringer containing $10 \mu\text{M}$ DALA. The right-hand column was obtained 40 min after the addition of $7 \mu\text{M}$ naloxone to the enkephalin-containing Ringer solution. The downward deflections represent responses to constant current hyperpolarizing pulses. The microelectrode was filled with 3 M potassium methylsulphate. The membrane potential of this neurone was -55 mV.

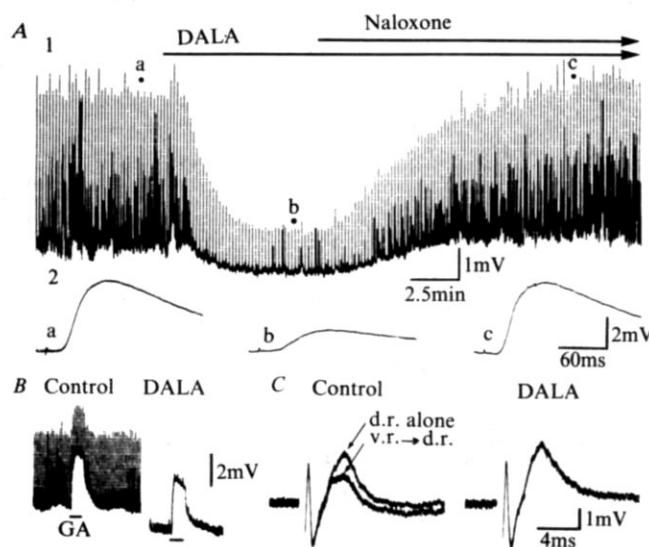


Fig. 6 Opioid peptide (DALA) blocks dorsal root potentials in the isolated frog spinal cord. **A**, shows a continuous recording with sucrose gap from the dorsal root. Dorsal root potentials (d.r.ps) (upward deflections) were evoked by stimulating a ventral root. 5 μM DALA greatly reduced the spontaneous and evoked d.r.ps. Naloxone (2 μM) reverses the effects of DALA. **A**₂ shows sample film records of the d.r.p. evoked at the times indicated by the small letters. **B** shows that DALA (10 μM) has no effect on the GABA sensitivity of the primary afferents at a time when it abolishes d.r.ps (upward deflections). The time scale is the same as in **A**. **C** shows superimposed film traces recorded with an extracellular electrode positioned in the dorsal horn. Stimulation of the dorsal root elicits a fast presynaptic fibre spike followed by a slow postsynaptic response (d.r. alone). Stimulation of the dorsal root 40 ms after a conditioning ventral root stimulus, at the peak of the d.r.p., results in a decreased postsynaptic response (v.r. → d.r.). DALA (10 μM) which completely abolished the d.r.p. in this preparation also blocks the decrease in the postsynaptic response.

interneurons, result in profound changes in the propagated output of principal cells.

In general, we can conclude that the cellular localization of neurotransmitter receptors may be more meaningful than their overall density in determining the relative importance of the neurotransmitter in a particular CNS region. In the present case, opioid peptides have physiological effects which would at first glance seem to be disproportionately large in comparison with

the modest concentration of endogenous peptides or opiate receptors. These effects can be accounted for by the findings that opiate receptors occur at strategic sites for the control of neuronal activity in the structures investigated. This emphasizes the importance of considering transmitter effects in the context of the intrinsic neuronal circuitry of a given CNS region rather than assessing the drug responsiveness of single neurones.

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1. Snyder, S. H. *Harvey Lect.* **79**, 291-314 (1979).
2. North, R. A. *Life Sci.* **24**, 1527-1546 (1979).
3. Jessell, T. M. & Iversen, L. L. *Nature* **268**, 549-551 (1977).
4. Mudge, A. W., Leeman, S. E. & Fischbach, G. D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 526-530 (1979).
5. Bornstein, J. C. & Fields, H. L. *Neurosci. Lett.* **15**, 77-82 (1979).
6. Konishi, S., Tsunoo, A. & Otsuka, M. *Nature* **282**, 515-516 (1979).
7. Nicoll, R. A., Siggins, G. R., Ling, N., Bloom, F. E. & Guillemin, R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2584-2588 (1977).
8. Zieglgänsberger, W., French, E. D., Siggins, G. R. & Bloom, F. E. *Science* **205**, 415-417 (1979).
9. Corrigan, W. A. & Linseman, M. A. *Can. J. Physiol. Pharmacol.* **57**, 1457-1459 (1979).
10. Dunwiddie, T., Mueller, A., Palmer, M., Stewart, J. & Hoffer, B. *Brain Res.* **184**, 311-330 (1980).
11. Gähwiler, B. H. *Neurosci. Abstr.* **5**, 282 (1979).
12. Jensen, R. A. *et al. Neurosci. Abstr.* **5**, 530 (1979).
13. Lee, H. K., Dunwiddie, T. & Hoffer, B. *Brain Res.* **184**, 331-342 (1980).
14. Robinson, J. H. & Deadwyler, S. A. *Neurosci. Abstr.* **5**, 281 (1979).
15. Stanley, J. C., DeFrance, J. F., Taber, K. & Dafny, N. *Neurosci. Abstr.* **5**, 282 (1979).
16. Fry, J. P., Zieglgänsberger, W. & Herz, A. *Brain Res.* **163**, 295-305 (1979).
17. Alger, B. E. & Nicoll, R. A. *Nature* **281**, 315-317 (1979).
18. Andersen, P., Eccles, J. C. & Løynning, Y. *J. Neurophysiol.* **27**, 608-619 (1964).
19. Alger, B. E. & Nicoll, R. A. *Brain Res.* (in press).
20. Katz, B. & Miledi, R. *Proc. R. Soc. B167*, 8-22 (1967).
21. Atweh, S. F. & Kuhar, M. J. *Brain Res.* **134**, 393-405 (1977).
22. Sar, M., Stumpf, W. E., Miller, R. J., Chang, K.-J. & Cuatrecasas, P. *J. comp. Neurol.* **182**, 17-38 (1978).
23. Jahr, C. E. & Nicoll, R. A. *Science* **207**, 1473-1475 (1980).
24. Nadi, N. S., Hirsch, J. D. & Margolis, F. L. *J. Neurochem.* **34**, 138-146 (1980).
25. Halasz, N., Ljungdahl, A. & Hokfelt, T. *Brain Res.* **154**, 253-271 (1978).
26. Price, J. L. & Powell, T. P. S. *J. Cell Sci.* **7**, 125-155 (1970).
27. Barker, J. L., Nicoll, R. A. & Padjen, A. *J. Physiol., Lond.* **245**, 521-536 (1975).
28. Schmidt, R. F. *Ergebn. Physiol.* **63**, 20-101 (1971).
29. Nicoll, R. A. & Alger, B. E. *Int. Rev. Neurobiol.* **21**, 217-258 (1979).
30. Nicoll, R. A. *J. Physiol., Lond.* **290**, 113-128 (1979).
31. Levy, R. A. *Prog. Neurobiol.* **9**, 211-267 (1977).
32. Suzue, T. & Jessell, T. *Neurosci. Lett.* **16**, 161-166 (1980).
33. Frenk, H., Urca, G. & Liebeskind, J. C. *Brain Res.* **147**, 327-337 (1978).
34. Henriksen, S. J., Bloom, F. E., McCoy, F., Ling, N. & Guillemin, R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5221-5224 (1978).
35. Krivoy, W. A. & Huggins, R. A. *J. Pharmac. exp. Ther.* **134**, 210-213 (1961).
36. Pomeranz, B. & Gurevich, N. *Eur. J. Pharmacol.* **60**, 307-313 (1979).
37. Corrado, A. P. & Longo, V. G. *Archs int. Pharmacodyn. Théor.* **132**, 255-269 (1961).

LETTERS

A soft X-ray halo around SU UMa

F. A. Cordova* & K. O. Mason†

* The University of California, Los Alamos Scientific Laboratory, Los Alamos, New Mexico 87545

† Space Science Laboratory and the Astronomy Department, The University of California, Berkeley, California 94720

The dwarf nova SU UMa was observed with the imaging proportional counter (IPC) detector¹ on the Einstein X-Ray Observatory on 4 October 1979 at 4 h UT for ~3,000 s. An X-ray source was detected at the position of the star. It had a flux of 1.3×10^{-11} erg cm⁻² s⁻¹ (0.6 counts per s) in the 0.1-4.5 keV energy range of the IPC, assuming a spectral temperature of ~10 keV (ref. 2). In addition, several areas of weaker emission were observed which form a ring that is symmetrically disposed about the position of the dwarf nova. This latter emission is detected only at energies below 0.7 keV. We discuss here the possibility that it arises in material that has been ejected from the star in a nova-like event.

An X-ray map of the SU UMa field is displayed in Fig. 1: the contours of equal X-ray intensity that are plotted show the relatively bright central source surrounded by a patchy distribution of enhanced X-ray emission. The mean radius of this 'ring' is ~14 arc min. The apparent knots of emission in the ring, of scale size ~1 arc min, are typically ~7 ± 3 counts above background. Additional structure may be present within the confines of the ring, particularly to the north-west of the dwarf nova. An examination of a large number of IPC fields demonstrates that this ring-like structure is unique to SU UMa and is not an instrumental effect.

To investigate the structure and luminosity of this feature further, we have summed the counts as a function of radius from the position of the central source in two energy bands. This is plotted in Fig. 2 which shows the counts accumulated in channels 1-4 and 5-14 of the 15 channel pulse-height analyser. Using the detector gain calculated from calibrations performed on the day of the observation, these channels encompass energy bands of 0.09-0.72 and 0.72-4.31 keV. The expected decrease of the counts as a function of radius from the centre of the field of view, caused by vignetting of the telescope, is shown by the dashed lines. The data in the higher energy band are distributed in a manner consistent with this function. However, at low energies

there is an excess above the vignetting curve at a radius of ~ 14 arc min, attributable to the ring. This feature thus seems to be most prominent at low energies.

The total number of counts in the knots comprising the ring (including the north-west filament) is $\sim 13\%$ that of the central source. This actually represents a lower limit to the ring intensity as much of the halo emission could come from extended regions whose surface brightness is too low to appear significant in the present data. Based on the lack of detection of the ring above ~ 0.7 keV, we estimate its temperature to be of the order of 10^6 K. Using this temperature we calculate the integrated flux from the ring to be $\sim 6 \times 10^{-13}$ erg cm $^{-2}$ s $^{-1}$, with an estimated uncertainty of 30%.

The symmetrical distribution of the ring around SU UMa suggests that it might be emission from material ejected by that object. It is unlikely that this material is radiatively excited by emission from the central star, as is the case in planetary nebulae, for example. The calculations of Tarter *et al.*³ allow us to estimate the temperature of a spherically symmetric, optically thin gas cloud irradiated by a central point source of X rays as a function of the parameter $\xi = L/nr^2$, where L is the luminosity of the central source, n is the gas density and r is the distance of the gas from the central star. At an assumed distance of 100 pc, $L \sim 1.5 \times 10^{31}$ erg s $^{-1}$ and $r \sim 0.4$ pc. Thus for SU UMa, $\xi \sim 1 \times 10^{-4} [(0.1 \text{ cm}^{-3})/n]$. This implies a temperature $< 10^4$ K, clearly inconsistent with the soft X-ray temperature observed in this observation.

Alternatively, we consider a mechanism analogous to that which gives rise to X-ray emission from Supernova remnants, that is, a blast wave from a nova explosion propagating through the interstellar medium (ISM) and shock heating the ambient material. If the density of the ISM is 0.06 cm^{-3} , which is appropriate for the galactic latitude of SU UMa⁴ ($b = 33^\circ$), the total amount of material swept up by a shock of radius 0.4 pc is $\sim 4 \times 10^{-4} M_\odot$. This is larger than the mass typically ejected in a nova explosion⁵ ($\sim 10^{-4} M_\odot$), so that it is reasonable to assume that the blast wave is in the adiabatic phase (see ref. 6). Adopting $T \sim 10^6$ K and a $0.2\text{--}2.0$ keV luminosity of $\sim 10^{30}$ erg s $^{-1}$ for the ring, the standard adiabatic solution gives a shock velocity, $V_s \sim 300 \text{ km s}^{-1}$, an initial explosion energy, $E_0 \sim 10^{45}$ erg, a density for the ISM, $n \sim 0.1 \text{ cm}^{-3}$, and an expansion age, $t \sim 500$ yr. The explosion energy required by the adiabatic model is

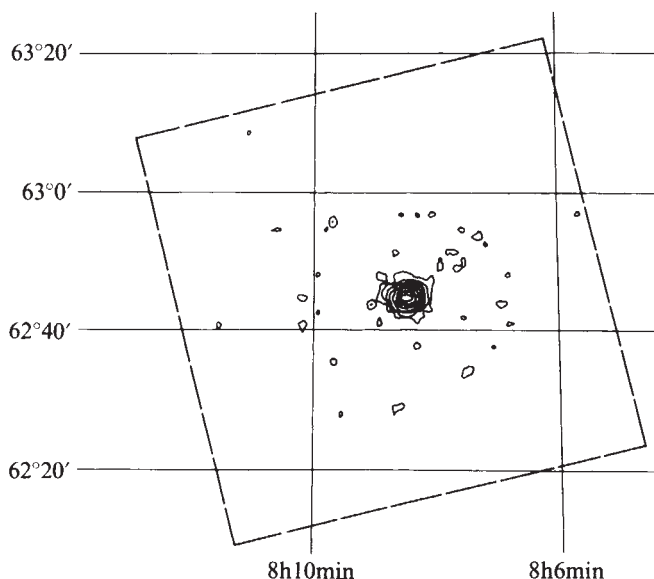


Fig. 1 The isointensity X-ray contour map of the $\sim 1^\circ \times 1^\circ$ IPC field (delineated by the dotted line boundary) centering on SU UMa. The size of the central object is consistent with the IPC spatial response to a point source (FWHM ~ 2.3 arc min). Centred approximately symmetrically around the central star at a radius of ~ 14 arc min is a nonuniform ring of emission.

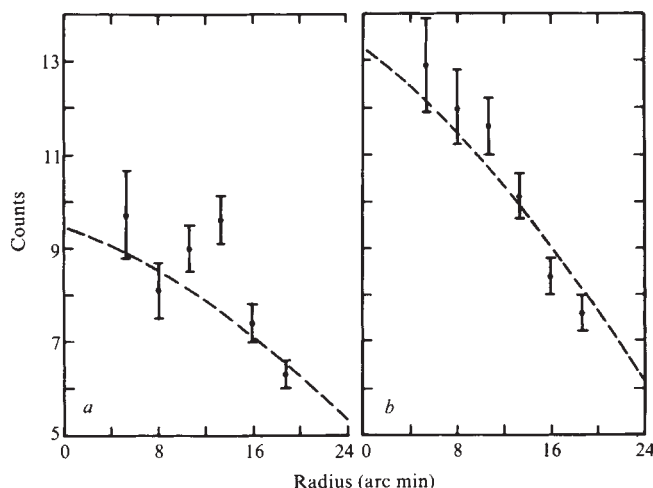


Fig. 2 The radial distribution of the intensity of the SU UMa field. The counts were integrated over radial rings in increments of 2.67 arc min extending outwards from the central source; the counts shown here have been normalized to a 2.67×2.67 arc min area: *a*, for the soft X-ray band of $0.1\text{--}0.7$ keV and *b*, for the hard X-ray band of $0.7\text{--}4.3$ keV. (The central source is not depicted as its count rate goes off scale.) The dashed lines represent the normalized effective area function of the IPG field (which depends on energy as well as spatial distribution); it also includes the vignetting function of the Einstein focusing telescope. Only in the soft X-ray band (*a*) is an enhancement above this function evident at the location of the ring.

similar to the energy liberated in a nova outburst⁷, while the ISM density derived is compatible with the expected value of 0.06 cm^{-3} (ref. 4). This interpretation of the X-ray ring is thus self-consistent.

A visual inspection of the region around SU UMa using the Palomar Sky Survey prints reveals no evidence for optical filamentary structure. The optical filaments associated with Supernova remnants are believed to be low temperature emission from relatively dense interstellar clouds^{8,9}. The lack of such emission is to be expected in the present case if the halo resides entirely in the intercloud medium, as is likely given its small size compared with the mean diameter and separation of interstellar clouds⁴.

The observation of an X-ray ring (that can be interpreted as resulting from a nova-like explosion) around an object that now shows the characteristics of a dwarf nova provides evidence for a connection between novae and dwarf novae. Other evidence that these two subclasses of cataclysmic variables may be related can be found in the fact that nova 1901 (GK Per) now exhibits dwarf nova-like behaviour¹⁰, and Nova Vul 1979 reportedly exhibited similar behaviour for at least 80 yr before its outburst¹¹. These findings may have important implications for evolutionary models of cataclysmic variables. As a possible first detection of an X-ray nova remnant, the X-ray ring around SU UMa provides a new parameter regime for exploring, as is done with Supernova remnants, the physical conditions in the interstellar medium and the physics of the nova outburst. A search for X-ray ejecta from other likely sources, including all types of cataclysmic variable stars, should also be made.

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1. Giacconi, R. *et al. Astrophys. J.* **230**, 540 (1979).
2. Cordova, F. A., Mason, K. O. & Nelson, J. E. *Astrophys. J.* (submitted).

3. Tarter, C. B., Tucker, W. H., & Salpeter, E. E. *Astrophys. J.* **156**, 943 (1969).
4. Allen, C. W. *Astrophysical Quantities* (Athlone, London, 1973).
5. Bath, G. T. & Shaviv, G. *Mon. Not. R. astr. Soc.* **183**, 515 (1978).
6. Culhane, J. L. in *Supernovae* (ed. Schramm, D. N.) 29 (Reidel, Dordrecht, 1977).
7. Robinson, E. L. *A. Rev. Astr. Astrophys.* **14**, 119 (1976).
8. McKee, C. F. & Cowie, L. L. *Astrophys. J.* **195**, 715 (1975).
9. Sgro, A. G. *Astrophys. J.* **197**, 621 (1975).
10. Webbink, R. V.S.S., R.A.S.N.Z. *Circ. No.* M78/5 (1978).
11. Liller, M. H. & Liller, W. *Astron. J.* **84**, 1357 (1980).

Simultaneous IR and X-ray burst observation of Ser X-1

Walter H. G. Lewin & Lynn R. Cominsky

Department of Physics and Center for Space Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Alistair R. Walker & Bruce S. C. Robertson

South African Astronomical Observatory, PO Box 9 Observatory, 7935 South Africa

It has been suggested^{1,2} that the very bright IR bursts^{1,3} observed from the Rapid Burster (MXB 1730–335) are due to Type I X-ray bursts⁴. When the bright IR bursts were detected, unfortunately there was no simultaneous X-ray coverage. Therefore, it is not known whether the IR bursts are associated with Type I or Type II bursts or with neither one. The suggestion^{1,2} is only based on the low recurrence rate of the IR bursts. If the suggestion is correct, similar bright IR bursts may, in general, accompany Type I X-ray bursts^{1,2}. This paper investigates this possibility for one specific case. During the 1977 coordinated worldwide burst watch⁵, simultaneous X-ray and IR observations were made. On UT June 17, 00 h 00 min 25s, a Type I X-ray burst was observed, which was almost certainly emitted by Ser X-1 (MXB 1837+05, 4U1837+04) (ref. 6). Simultaneous IR observations failed to detect any bursts. We report here on these observations.

Between UT June 16, 20 h 40 min and June 17 02 h 30 min, IR observations were made with the SAAO 30-inch telescope in Sutherland. The field of view was approximately centered on the star which was first identified by Davidson⁷. It is now known that the actual star is ~ 2.2 arc s away⁸, but that is of no consequence here since the radius of the field of view was ~ 35 arc s. A Glass MK2 IR photometer was used in combination with a lead sulphide detector. The observations were made at $2.2 \mu\text{m}$ using a Johnson K filter (bandwidth $\sim 0.48 \mu\text{m}$). The output was displayed on a chart recorder running at sufficient speed that the lock-in time constant of 1 s was the dominant smoothing factor. No indication of an IR burst was found. A 3σ signal in a 1-s readout corresponds to a peak K magnitude of ~ 7 . Therefore, at a 3σ level of confidence, the IR flux integrated over the 20 s duration of the burst was less than $1.3 \times 10^{-9} \text{ erg cm}^{-2}$.

SAS-3 observed Ser X-1 (MXB 1837+05, 4U1837+04) between UT 1977 June 11.6 and June 17.5. Between June 11.6 and 14.9 the source was observed with the Horizontal Tube (HT) system^{9,10}. A total of nine Type I bursts were observed. From June 14.9 to June 17.5 the source was observed with the Center Slat (CS) system^{9,10} (field of view $\sim 1.0^\circ \times 30^\circ$ FWHM) and a total of six bursts were observed. When a Type I X-ray burst occurred on June 17 00h 00 min 25 s, Ser X-1 was within a few arc min of the centre line of the CS and 9.8° from the spacecraft equator (elevation). Consequently, at the time of the burst, the collimator transmission function was 0.57 ± 0.03 . The rise time of the burst was less than 1 s, the burst decayed gradually and lasted for about 20 s. The integrated burst flux, in the range 1.4–14 keV, was $(9.3 \pm 0.9) \times 10^{-8} \text{ erg cm}^{-2}$.

There is a chance that the X-ray burst did not come from Ser X-1; although it is not possible to calculate a formal probability, we believe this chance to be small. The burst was only observed in the CS and not in the HT system^{9,10} ($\sim 1.7^\circ$ FWHM). Thus, it could only come from sources viewed by the CS excluding the

area of overlap with the HT system. This excludes the burst source Aql X-1 (ref. 11) since it was located near the centre of the HT system. The only known burst source other than Ser X-1 in the field of view of the CS system was Aql MXB (ref. 9). However, only 15% of the error box of Aql MXB was observed by the CS (and not by the HT system) and the burst frequency of Aql MXB is very low; only one burst was observed in 1976 in 12 days of observing⁹. In addition, a reanalysis at MIT of the 1976 SAS-3 data showed that the burst from Aql MXB (ref. 9) could have come from Aql X-1 (L. Cominsky, A. Lawrence and W. Lewin; unpublished data). If the burst sources Aql X-1 and Aql MXB are the same source, as we suspect, then Ser X-1 was the only known burst source in the field of view of the CS and not in the HT system.

There is another reason why we believe that the burst observed in the CS system was emitted by Ser X-1. Ser X-1 was burst active during the observations with the HT system from June 11.6 to 14.9 and the burst observed on June 17 00 h 00 min 25 s with the CS system was very similar to many from Ser X-1 observed a few days earlier with the HT system.

The ratio of the observed IR ($2.2 \mu\text{m}$, $0.48 \mu\text{m}$ bandwidth) and X-ray (1.4–14 keV) fluxes for the burst from Ser X-1 is less than 1.4×10^{-2} at a 3σ level of confidence. For stars in the direction of Ser X-1 ($l^{\text{II}} \sim 36^\circ$, $b^{\text{II}} \sim 5^\circ$), the colour excess $E_{B-V} \sim 0.6 \text{ mag kpc}^{-1}$ (ref. 12). With a scale height of $\sim 150 \text{ pc}$ (ref. 13), the path through the dust layer along the line of sight to Ser X-1 is $\sim 1.8 \text{ kpc}$. Thus, $E_{B-V} \sim 1.1$ which yields corresponding values for the extinction¹³ $A_V \sim 3.3 \text{ mag}$ and $A_K \sim 0.3 \text{ mag}$. Thus, after correction for extinction, we find for the ratio of IR and X-ray fluxes an upper limit of 1.8×10^{-2} (3σ level of confidence). We will compare this with the flux ratios of IR bursts and Type I X-ray bursts (not observed simultaneously) from the Rapid Burster.

From the work by Kulkarni *et al.*¹ ($1.6 \mu\text{m}$, bandwidth $0.3 \mu\text{m}$) and that of Jones *et al.*³ ($2.2 \mu\text{m}$, bandwidth $0.4 \mu\text{m}$), we calculated the integrated IR fluxes. The six IR bursts observed by the Indian group had an average duration of $\sim 35 \text{ s}$ and an average burst flux integrated over the duration of the bursts of $\sim 2.2 \times 10^{-8} \text{ erg cm}^{-2}$. Jones *et al.* observed two events and measured burst durations of $\sim 20 \text{ s}$ and $\sim 12 \text{ s}$. The integrated IR flux values were $\sim 8.2 \times 10^{-9} \text{ erg cm}^{-2}$ and $\sim 5.5 \times 10^{-9} \text{ erg cm}^{-2}$.

Using SAS-3 data (not taken simultaneously with the IR observation), we calculated the integrated fluxes of three Type I X-ray bursts of $\sim 20 \text{ s}$ duration from the Rapid Burster. All three bursts were observed with the HT system (1.7° FWHM) and in all cases, the Rapid Burster was within 0.3° of the centre of the field of view. The average value (corrected for aspect) in the 1.3–18 keV interval is $(1.0 \pm 0.1) \times 10^{-7} \text{ erg cm}^{-2}$.

Thus we find that the observed IR/X-ray (Type I) flux ratios are ~ 0.22 using the IR data by Kulkarni *et al.*¹ (H band) and ~ 0.07 (average value for the two events) using the IR data by Jones *et al.*³ (K band). For the X-ray flux we used the SAS-3 data which were not taken simultaneously. After correction for extinction^{13,14} ($A_H \sim 2.0$; $A_K \sim 1.1$), these flux ratios become ~ 1.38 and ~ 0.19 respectively. Thus they are respectively ~ 77 times and ~ 10 times larger than the 3σ upper limit reported in this paper for the Ser X-1 burst.

Optical bursts have been observed simultaneously with Type I X-ray bursts^{15–18} from three burst sources, including Ser X-1 (ref. 17). The optical emission is believed to be due to X-ray heating of the accretion disk^{16,17,19–21}. The disk is expected to radiate more or less as a black body of different temperatures whose emission reaches a maximum at energies considerably above those of visible photons. An extrapolation of its spectrum to the IR would predict flux levels several orders of magnitude below what would be detectable. Therefore, the fact that IR bursts were detected from the Rapid Burster excludes that they are related to optical bursts as observed in three other burst sources. If they are of thermal origin, the IR brightness temperatures would be in excess of $4 \times 10^{10} \text{ K}$ (ref. 3). It has been suggested that the IR fluxes are produced by maser action².

The purpose of this paper is to see whether unusually strong IR fluxes as detected from the Rapid Burster (whose origin is still uncertain) accompany Type I X-ray bursts from Ser X-1. Our upper limit reported here indicates that the Type I burst from Ser X-1 was not accompanied by a bright IR burst. One could perhaps argue that the IR bursts are highly directional and that in the case reported here, for Ser X-1, the IR burst missed the Earth. However, if the IR beams are highly directional, they could not be associated with Type I X-ray bursts from the Rapid Burster. Since the Type I X-ray bursts from the Rapid Burster, when present, occur on time scales of hours, one would expect to detect IR bursts (if narrowly beamed) at a much lower rate. However, eight (of which six came in pairs) were detected in 7.9 h (refs 1, 3).

We conclude that if the Type I X-ray burst observed on UT June 17, 00 h 00 min 25 s came from Ser X-1, as is very likely, the possibly associated $2.2 \mu\text{m}$ IR integrated burst flux (K band) was less than $1.3 \times 10^{-9} \text{ erg cm}^{-2}$ (3σ level of confidence). This is substantially fainter than IR bursts observed from the Rapid Burster. However, the integrated fluxes observed in Type I X-ray bursts from the Rapid Burster and Ser X-1 are comparable. Thus, if Type I X-ray bursts are associated with the IR bursts, as suggested^{1,2} the flux ratio IR/X-ray is substantially (one to two orders of magnitude) lower for the June 17, 1977 burst from Ser X-1 than for the Rapid Burster.

We suspect that Type I X-ray bursts are not associated with bright IR bursts and that the Rapid Burster is unique in producing the IR bursts, as it is unique in many other ways. If the IR bursts are associated with the Type II bursts from the Rapid Burster, the IR bursts must be highly anisotropic since their frequency of occurrence is considerably less than that of the Type II bursts^{20,21}.

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- Kulkarni, P. V., Ashok, N. M., Apparao, K. M. V. & Chitre, S. M. *Nature* **280**, 819 (1979).
- Apparao, K. M. V. & Chitre, S. M. (in preparation).
- Jones, A. W. *et al. Nature* **283**, 550 (1980).
- Hoffman, J. A., Marshall, H. L. & Lewin, W. H. G. *Nature* **271**, 630 (1978).
- Lewin, W. H. G. *IAU Circ.* No. 3078 (1977).
- Li, F. K. *et al. Mon. Not. R. Astr. Soc.* **179**, 21P (1977).
- Davidson, A. *IAU Circ.* No. 2824 (1975).
- Thorstensen, J., Charles, P. & Bowyer, S. *IAU Circ.* No. 3253 (1978).
- Lewin, W. H. G. *et al. Mon. Not. R. Astr. Soc.* **177**, 93P (1976).
- Buff, J. *et al. Astrophys. J.* **212**, 768 (1977).
- Oda, M. *IAU Circ.* No. 3481 (1980).
- Lucke, P. B. *Astr. Astrophys.* **64**, 367 (1978).
- Allen, C. W. *Astrophysical Quantities* (Athlone, London, 1964).
- Kleinmann, D., Kleinmann, S. & Wright, E. *Astrophys. J. Lett.* **210**, L83 (1976).
- Grindlay, J. E. *et al. Nature* **274**, 567 (1978).
- McClintock, J. E. *et al. Nature* **279**, 47 (1979).
- Hackwell, J. A. *et al. Astrophys. J.* **233**, L115 (1979).
- Pedersen, H. *et al. IAU Circ.* No. 3399 (1979).
- Canizares, C., McClintock, J. E. & Grindlay, J. *Astrophys. J.* **234**, 556 (1979).
- Lewin, W. H. G. in *X-Ray Astronomy* (eds Baity, W. & Peterson, L.) 133 (Pergamon, Oxford, 1979).
- Lewin, W. H. G. & Clark, G. W. *Ann. N.Y. Acad. Sci.* **336**, 451 (1980).

Intracrystalline grafting on layer silicic acids

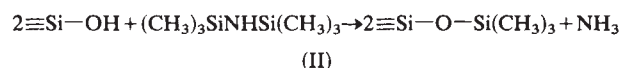
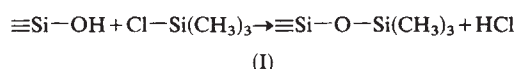
E. Ruiz-Hitzky & J. M. Rojo

Grupo de Físico-Química Mineral, Consejo Superior de Investigaciones Científicas, Serrano 115 dpdo., Madrid-6, Spain

Silanol groups (Si—OH) present at the surface of amorphous silica and microcrystalline silicates can react with organosilicon compounds to form stable organic derivatives¹⁻³. The grafting reaction always occurs at the external surfaces of these materials. The present work considers the intracrystalline grafting on

internal Si—OH groups in certain layer silicic acids. For the reaction to occur, the silicic acid must have been expanded by the intercalation of appropriate organic polar molecules such as dimethylsulphoxide; in this way, the individual layers of the silicic acid can be separated and the internal Si—OH groups become accessible to the grafting reagents. The resulting grafted products are organosilicic compounds which retain the lamellar structure of the starting silicic acids, but with the interlamellar surfaces covered by organic groups homogeneously distributed. These materials can be thought of as planar silicones.

Starting materials for the reaction are crystalline silicic acids obtained from natural or synthetic alkali layer silicates by acid treatment^{4,5}. These silicic acids have a lamellar structure with intracrystalline Si—OH groups covering the interlamellar surfaces. $\alpha\text{-H}_2\text{Si}_2\text{O}_5$, $\text{H}_2\text{Si}_8\text{O}_{17} \cdot n\text{H}_2\text{O}$ and $\text{H}_2\text{Si}_4\text{O}_{29} \cdot n\text{H}_2\text{O}$ are used in this work. The last silicic acid is called H-magadiite because it was prepared by acid treatment of the mineral magadiite⁴. Reagents used in the present investigation were trimethylchlorosilane (I) and hexamethyldisilazane (II) which react with the Si—OH groups of the mineral substrate to form siloxane bridges (Si—O—Si):



Mixtures of both reagents can be also used with positive results.

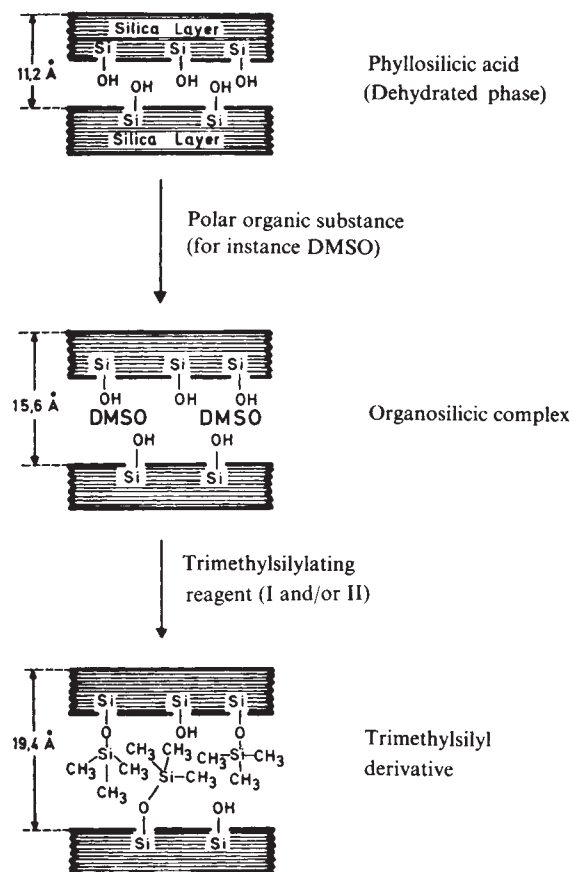


Fig. 1 Interlamellar grafting of $-\text{Si}(\text{CH}_3)_3$ groups. The synthesis of the trimethylsilyl derivative of the phyllosilicic acids was carried out in two steps: first, the starting silicic substrate (dehydrated phase) was added to an excess of an organic polar substance, as DMSO, and the mixture was stirred continuously for 72 h; excess DMSO was eliminated and the organosilicic complex was recovered. In the second step, reaction between this complex and the trimethylsilylating reagents (I and/or II) was carried out in dioxane for time periods ranging from 24 to 48 h at reflux temperature.

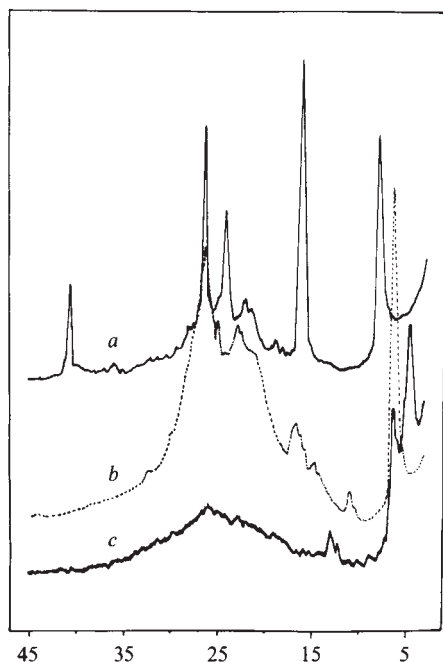


Fig. 2 X-ray diffraction patterns of: *a*, H-magadiite silicic acid, pretreated at 100 °C, 24 h; *b*, H-magadiite (*a*) treated by NMF (intermediate *a*/NMF complex); *c*, trimethylsilyl derivative of *a*, obtained by refluxing the *a*/NMF complex with a mixture of I and II reagents.

If the grafting reaction is carried out by direct treatment of the mineral substrate with organosilanes (I) and/or (II), the resulting product is an organosilicic material with $(\text{CH}_3)_3\text{Si}-$ groups bonded only at the external surfaces of the mineral particles. However, if the silicic acid is previously treated with a polar organic substance such as dimethylsulphoxide (DMSO), *N*-methylformamide (NMF), or *N,N*-dimethylformamide, and subsequently reacted with (I) and/or (II) reagents, then an interlayer grafting on the internal $\text{Si}-\text{OH}$ groups is produced. The intercalation of the above polar organic substances into the interlayer space of the phyllosilicic acids is a reversible process, as described by Lagaly *et al.*⁵. The swelling produced by these organic molecules makes the internal $\text{Si}-\text{OH}$ groups accessible to the trimethylsilylating reagents, so that subsequent grafting reaction can occur. Figure 1 explains the different steps in the synthesis of the organosilicic materials.

Evidence of the interlayer grafting of the $-\text{Si}(\text{CH}_3)_3$ groups was obtained from X-ray diffraction diagrams. The basal spacing (d_{001}) of the phyllosilicic acids increased in all cases, with a mean value of $\Delta d = 8.2 \text{ \AA}$, corresponding to the thickness of two

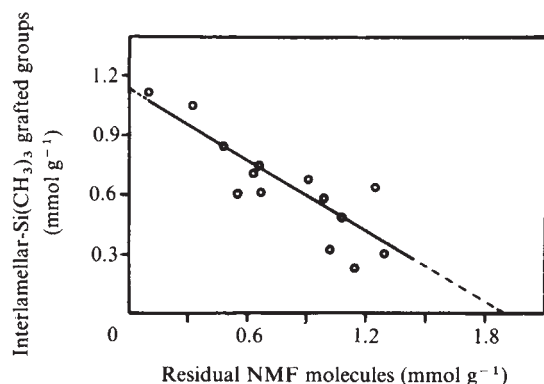


Fig. 3 Variation in the quantity of $-\text{Si}(\text{CH}_3)_3$ groups grafted into the interlamellar space of H-magadiite, as a function of the quantity of *N*-methylformamide (NMF) residual molecules.

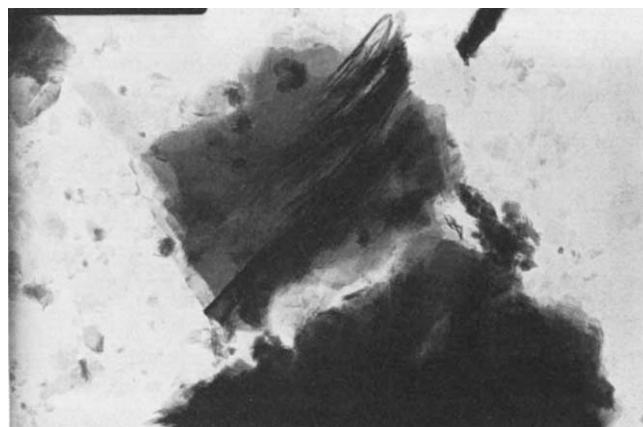
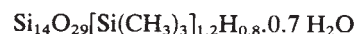


Fig. 4 Microphotograph of a trimethylsilyl derivative of the H-magadiite phyllosilicic acid. Magnification $\times 75,000$.

$-\text{Si}(\text{CH}_3)_3$ groups (Table 1). A disorder in the stacking of the lamellae of the silicic acids is produced during the grafting reaction, as indicated by the broadness of the peaks in the X-ray diffraction pattern (Fig. 2).

Chemical analysis and IR spectroscopy show that the progress of the grafting reaction is accompanied by the displacement of the polar organic molecules previously intercalated. The kinetic data show that the rate of grafting reaction of $-\text{Si}(\text{CH}_3)_3$ groups is equal in absolute value to the rate of desorption of organic polar molecules. Depending on the experimental conditions, a variable quantity of these organic molecules can be trapped in the interlamellar space of the resulting product. The quantity of residual organic molecules (NMF in this case) decreases with the number of $-\text{Si}(\text{CH}_3)_3$ grafted groups according to a linear relationship (Fig. 3).

The surface covered by grafted $-\text{Si}(\text{CH}_3)_3$ groups and NMF residual molecules has, in all cases, a constant area equivalent to the calculated area of the interlamellar surface. Chemical analysis and titration of the residual $\text{Si}-\text{OH}$ groups by NaOH solution can be used to obtain the composition of the resulting materials. For example, in a trimethylsilyl derivative of H-magadiite with a 40% of residual $\text{Si}-\text{OH}$ groups, the resulting formula can be written as:



where the residual content of organic polar molecules (NMF), as determined by chemical analysis, has been subtracted.

The high thermal stability of these organosilicic materials has been determined by DTA technique. The $-\text{Si}(\text{CH}_3)_3$ grafted groups remain unaltered until 400 °C.

No important textural changes are observed by electron microscopy when the phyllosilicic acids have been grafted. The planar morphology of the resulting organosilicic materials is shown in Fig. 4, where some corrugation can be seen which could be attributed to the effect of the $-\text{Si}(\text{CH}_3)_3$ groups grafted on the surfaces of the lamellae.

Table 1 Effect of the interlamellar $-\text{Si}(\text{CH}_3)_3$ grafting groups on the basal spacing of some typical phyllosilicic acids

Phyllosilicic acid (dehydrated phase)	d_{001} ($\text{\AA}$)	Trimethylsilyl derivative	
		d_{001} ($\text{\AA}$)	Δd ($\text{\AA}$)
$\alpha\text{-H}_2\text{Si}_2\text{O}_5$	7.6	16.2	8.6
$\text{H}_2\text{Si}_8\text{O}_{17}$	16.5	24.4	7.9
$\text{H}_2\text{Si}_{14}\text{O}_{29}^*$	11.2	19.4	8.2

* H-magadiite.

The results described above show that it is possible to obtain a new family of macromolecular organosilicon compounds, in which the chemical composition depends both on the nature of the starting materials and on the experimental conditions. One feature of these materials is that they maintain the layer structure of the original silicic substrate, but have surface properties corresponding to the organic groups attached, for example, they exhibit a marked organophilic character.

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1. Armistead, C. G. & Hockey, J. A. *Trans. Faraday Soc.* **63**, 2549–2556 (1967).
2. Hair, M. L. & Herli, W. J. *phys. Chem.* **73**, 2372–2378 (1969).
3. Ruiz-Hitzky, E. & Fripiat, J. J. *Clays Clay Miner.* **24**, 25–30 (1976).
4. Eugster, H. P. *Science* **157**, 1177–1180 (1967).
5. Lagaly, G., Beneke, K. & Weiss, A. Z. *Naturforsch.* **28b**, 234–238 (1973); *Am. Miner.* **60**, 650–658 (1975).

An electrical model of the crust and upper mantle in Scotland

V. R. S. Hutton, M. R. Ingham & E. W. Mbipom

Department of Geophysics, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JZ, UK

Magnetotelluric and magnetovariational studies in southern Scotland^{1–4} have been followed by deep electrical soundings at an additional 30 sites. These lie on an approximately linear traverse extending from Kinlochbervie in the Lewisian Foreland to Borthwickbrae in the Scottish Borders (Fig. 1a). This traverse crosses the main geological zones of Scotland and follows reasonably closely the line of the LISP⁵ seismic project (Fig. 1b). Full details of these geo-electric studies will be published elsewhere. We present here results of two-dimensional modelling of both the magnetotelluric and geomagnetic response functions^{6,7} obtained along the traverse.

The model was derived as follows. The digital data from each site were analysed in similar ways to those adopted in refs 8–10. The resulting magnetotelluric response functions, apparent resistivity and phase, were found to be anisotropic at all sites. This indication of lateral variations in conductivity structure was supported by the behaviour of the geomagnetic response functions.

A one-dimensional inversion¹¹ of the average apparent resistivity and phase data was obtained for each site. These were then used as the basis for applying a two-dimensional modelling programme¹² to each of the sections AA' and BB' of the total traverse.

Successive adjustments were then made to the initial two-dimensional models to obtain models which fitted not only both the maximum and minimum apparent resistivity data but also the geomagnetic response functions.

Finally, due to the similarity in the two best-fitting models in the region of overlap to the north of the Highland Boundary Fault, a single geo-electric model was constructed to represent the complete traverse. Observations at Newcastleton and Towhouse¹¹ have been taken into account to provide a very provisional model across the probable region of the Iapetus suture.

The resulting geo-electric model is given in Fig. 2a. This model provides reasonably good agreement between observed and computed response functions for the sites along the traverse marked by arrows in Fig. 1a. For traverse AA', the modelling has been discussed by Mbipom¹³ and for traverse BB' a similar account is in preparation. Here we compare examples of observed and model responses (Figs 3, 4). The model suggests that the electrical structure of the crust and upper mantle in Scotland is, as one might expect from the surface geology, very complex and that there seems to be a distinctive conductivity-depth profile

associated with each of the major geological zones along the traverse. For example, the Precambrian fragment to the west of the Moine Thrust is characterized by a resistive crust overlying a less resistive upper mantle, while in the Caledonian metamorphic belt, only the upper 10–15 km of the crust is resistive and the lower crust and uppermost mantle are relatively conducting. The major granitic intrusions are particularly resistive and where the observational density is sufficient the electrical model seems to delineate their form with some success. At the Great Glen, there is a 10 km wide gap in the resistive upper crust where an upper conducting layer connects with the deeper conducting layer of the lower crust and upper mantle. Above average crustal conductivity is also found at shallow depths near the Highland Boundary, Southern Uplands and Stobick Faults. The section of this two-dimensional model covering the south of Scotland confirms and extends the previous one-dimensional interpretation of this region⁴. In the Southern Uplands, the crust is resistive to a depth of ~20 km, and overlies a highly conducting layer, $\sigma \approx 2 \times 10^{-2} \text{ S m}^{-1}$, of poorly defined thickness, 30–70 km. Observational data from the Midland Valley and Northumberland Basin are sparse but in both regions a good conductor exists in the upper crust and it overlies a more resistive layer with the interface between them shelving rapidly near the boundaries of these regions.

This Scottish electrical model can be compared with deep electrical soundings in other countries. Jones and Hutton⁴ have already noted the similarity in the conductivity-depth profiles in

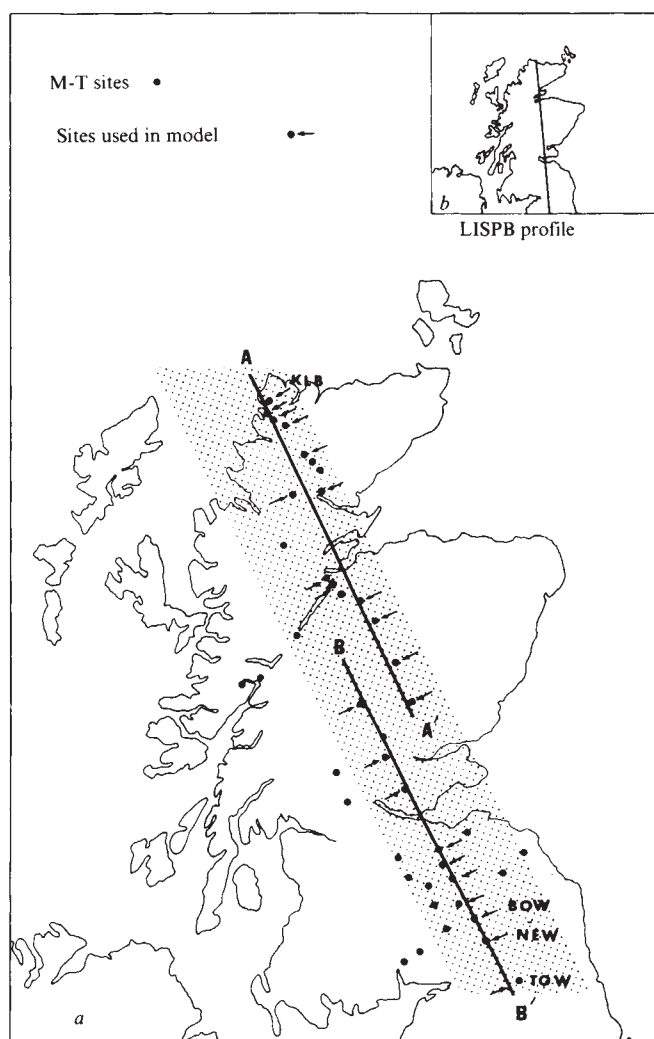


Fig. 1 a, Map of Scotland showing the location of the magnetotelluric (M-T) sites. KLB, Kinlochbervie; BOW, Borthwickbrae; NEW, Newcastleton; TOW, Towhouse. b, Map of Scotland showing the line of the LISP study.

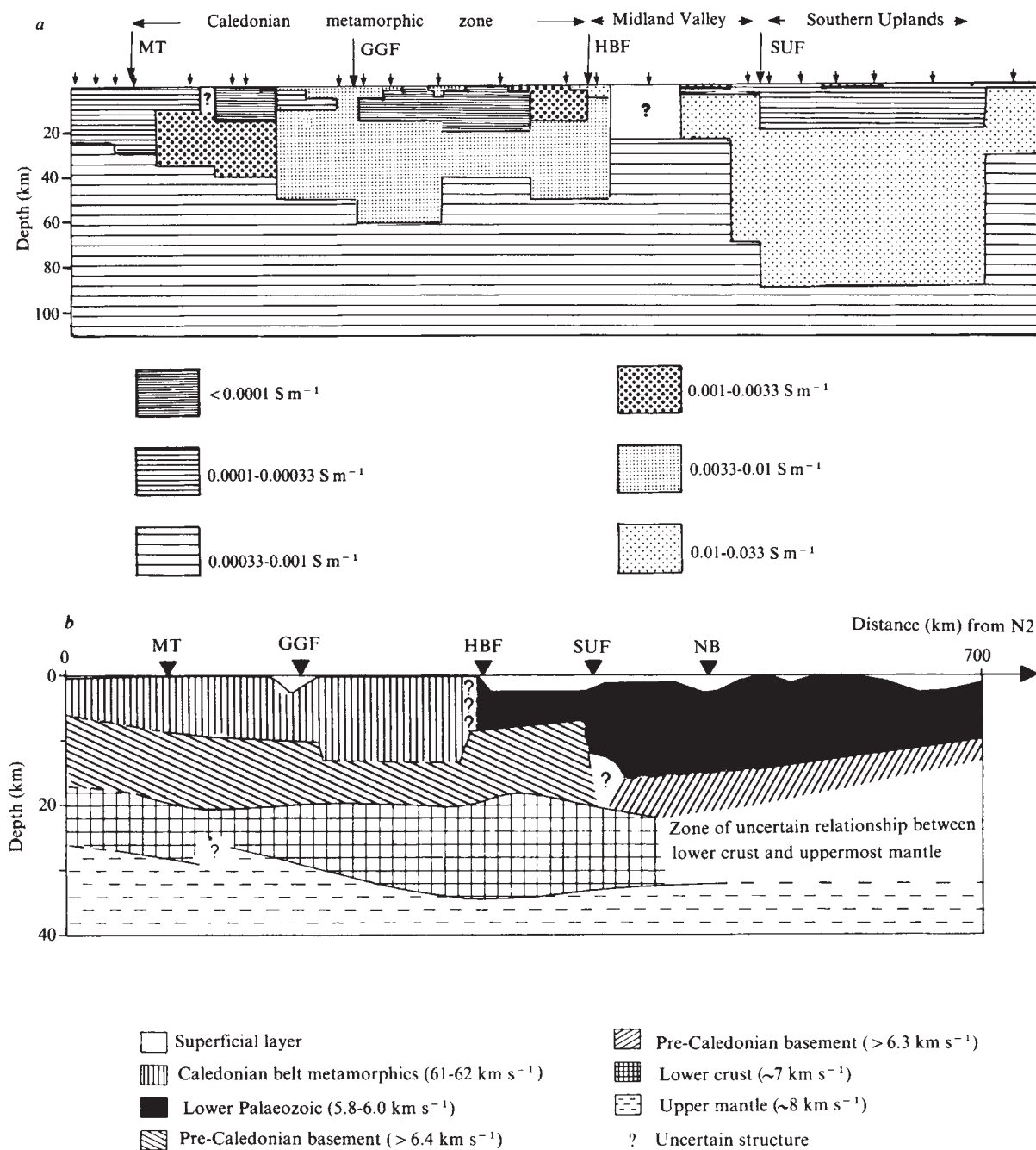


Fig. 2 *a*, The electrical conductivity model of the crust and upper mantle in Scotland for the traverse indicated by the shaded zone in Fig. 1*a*. *b*, Schematic seismic cross-section through the crust and uppermost mantle of northern Britain. MT, Moine Thrust; GGF, Great Glen Fault; HBF, Highland Boundary Fault; SUF, Southern Uplands Fault; NB, Northumberland Basin. (Figure 2*b* from ref. 5.)

the Southern Uplands and in the Canadian Appalachians, regions of common geological history. Further comparison of the Scottish model and that obtained from the Canadian study¹⁴ for a traverse from the Grenville Province of the Canadian Shield to the Appalachians shows another common feature. The electrical conductivity of the lower crust in the neighbourhood of the St Lawrence river, that is the Logan line, where the basement was formed during the Grenville orogeny, is similar to that around the Great Glen, $5 \times 10^{-3} \text{ S m}^{-1}$. In fact, a conductivity-depth profile consisting of a resistive upper crust, a more conducting lower crust and uppermost mantle and a relatively resistive upper mantle seems to be the most common profile in stable continental regions¹⁵⁻²⁴.

The average conductivity of near-surface continental crustal rocks is generally accepted to be in the range²⁵ 10^{-5} to 10^{-3} S m^{-1} , as found in the Scottish study along much of the

traverse. In some locations, however, the conductivity within the top few kilometres is as high as 10^{-2} to 10^{-1} S m^{-1} . Conductivities of this order of magnitude have been found to be associated with the presence of graphitic schists, geothermal prospects or highly conducting fluids in loosely consolidated sediments.

Thus, in general, all sections of the Scottish model have their counterparts elsewhere. The lateral variation in structure over a distance of only 400 km is, however, probably more striking than in any previous study.

The only published model of the deep structure across Scotland, other than those inferred from studies of surface rocks, is that provided by the LISPB seismic study (Fig. 2*b*).

There is only partial agreement between the geometries of the seismic and electric models. For example, in the Lewisian, the boundary between the resistive crust and more conducting

upper mantle occurs at a depth which corresponds well with the Moho as deduced from the seismic study. Further south, however, there is no boundary in the electrical model at Moho depths, but rather a boundary between the resistive upper crust and the more conducting lower crust. This boundary agrees reasonably well with the depth of the Conrad discontinuity. Sharp changes in depth of seismic boundaries, for example, at the Great Glen Highland Boundary and Southern Uplands Faults have their counterparts in the electrical model. The existence of a resistive crust of thickness about 20 km in the Southern Uplands is compatible with the absence of a seismic horizon at 10–15 km depth and a very good conductor in the uppermost mantle is compatible with the absence of a clearly defined signal from Moho depths.

Oxburgh *et al.*²⁶ have used a schematic crustal model to explain their UK heat flow data. They propose that the variation in heat flow is due to variation in crustal heat production, with the higher heat flow belts including a higher proportion of granites than elsewhere. They require the granites to have a thickness of 16.6 km which is in reasonable agreement with that indicated for the Cairngorm granites in the geoelectric model.

The interpretation of the electrical model will be discussed in detail elsewhere. Here we briefly consider possible explanations of anomalously high conductivity. In the upper crust, it can arise, primarily, as a result of electrolytic conduction in pore fluids, of abnormally high temperature or of intense mineralization. Note that, along the Scottish traverse, the most conducting surface layers coincide with known sedimentary basins in which above average heat flow has recently been reported²⁶. The relative contribution of porosity, salinity and temperature in these regions could thus be of interest to the UK geothermal programme. Moreover, similar electrical soundings might be useful to current studies of the deep sedimentary basins in England, especially as pore fluid data are already available from those regions²⁷.

In the lower crust, the increased pressure and hence crack closure make it unlikely that conduction in electrolytic pore fluids can account for the presence of a conducting zone at this depth. Solid conduction in dry rocks also seems improbable as extremely high temperatures—greater than 700 °C—would be required to provide conductivities of the order of magnitude found in this study. On the other hand, solid conduction through hydrated rocks may be a possibility. Laboratory measurements have also indicated that time-dependent increases in conductivity of up to four orders of magnitude can occur in the solid state due to phenomena such as order–disorder in crystals and rocks²⁸. The presence of hydrated rocks with or without associated partial melting has, nevertheless, been the most frequent interpretation for conducting layers in the lower crust^{15–24}.

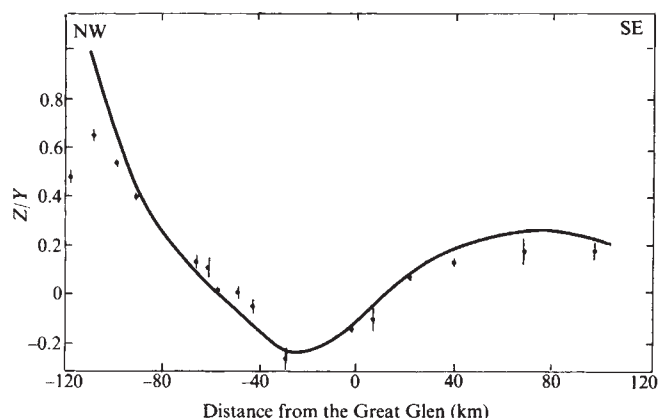


Fig. 3 The variation across the traverse AA' of the vertical magnetic field transfer function Real (Z/Y), that is the E-polarization case, for a period $T = 95$ s. The solid curve represents the values computed for the model given in Fig. 2a. The dots and associated error bars refer to values computed from field data.

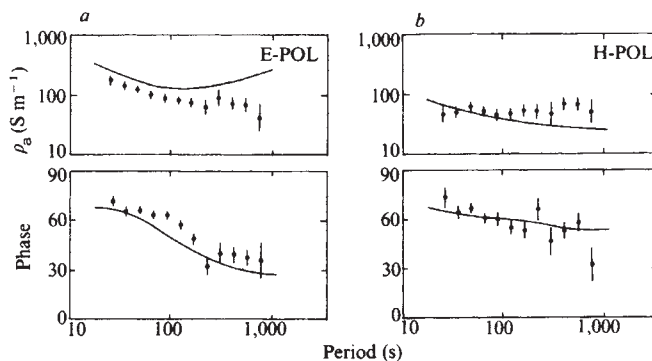


Fig. 4 Apparent resistivity and phase versus period data and associated errors for Yarrow in the Southern Uplands—the station on the traverse BB' immediately north of BOW in Fig. 1. Curves *a* and *b* correspond respectively to the E and H polarization cases and the smooth curves to the computed values for the model given in Fig. 2a.

For example, Berdichevsky *et al.*¹⁸ have discussed the increase in electrical conductivity of granite and other rock types on melting in the presence of water at temperatures of 400–600 °C. They have associated the zone of active granitization and melting with the transition zone between amphibolitic and granulitic facies, that is with the Conrad discontinuity, with the necessary few per cent of water being released as a result of the dehydration at this boundary. Stanley *et al.*²³ have used laboratory results^{29–32} to argue that hydrated rocks, such as granite, tonalite, syenite and gabbro, in the upper crust will start melting at 500 °C, especially where the rocks have been subjected to rapid alteration processes. The 500 °C isotherm is, in this case, coincident with a large change in electrical conductivity.

The interpretation of the lower crustal conductor in Scotland in terms of hydrated rocks and partial melting and the other interpretations will be considered elsewhere. However, note that if the granites in Scotland are uranium enriched as in Cornwall and northern England, then a temperature gradient of 30 °C km⁻¹ and a depth to the 500 °C geotherm of ~16 km can be expected.

In consideration of Fig. 2a, note that: there can be some trade-off between interface depth and conductivity; use of closer station spacing and broader-band electrical soundings may introduce significant changes to the model in some regions; and careful assessment of the validity of laboratory studies is required before further conclusions can be drawn.

It is unfortunate for the tectonic implications of the model that more electrical soundings have not been undertaken in the Midland Valley. If, however, the major boundaries in conductivity structure now suggested to the north and south of this region were confirmed an interpretation of these lateral variations would be possible in terms of former subduction zones as has been suggested in similar studies elsewhere (see refs 33–35). The location of a subsequent subduction zone and the nature of the Iapetus suture³⁶ may become clearer on the completion of current analyses of geomagnetic and geo-electric observations from northern England.

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1. Jain, S. & Wilson, C. D. V. *Geophys. J.R. astr. Soc.* **12**, 165–180 (1967).
2. Edwards, R. N., Law, L. K. & White, A. *Phil. Trans. R. Soc.* **270**, 289–323 (1971).
3. Hutton, V. R. S. & Jones, A. G. *J. Geomagn. Geoelec.* (in the press).
4. Jones, A. & Hutton, R. *Geophys. J.R. astr. Soc.* **56**, 329–368 (1979).
5. Bamford, D., Nunn, K., Prodehl, C. & Jacob, B. *Geophys. J.R. astr. Soc.* **54**, 43–60 (1978).
6. Schmucker, U. *Bull. Scripps Inst. Oceanogr.* **13**, 1–165 (1970).
7. Banks, R. J. *Phys. Earth planet. Inter.* **7**, 339–348 (1973).
8. Hermance, J. T. *Phys. Earth planet. Inter.* **7**, 349–364 (1973).
9. Haak, V., *Bayer. Akad. Wissen.* **158**, 1–105 (1978).
10. Rooney, D. & Hutton, V. R. S. *Geophys. J.R. astr. Soc.* **51**, 91–119 (1977).
11. Jones, A. G. thesis, Univ. Edinburgh (1977).
12. Brewitt-Taylor, C. & Johns, P. B. *J. Geomagn. Geoelec.* (in the press).
13. Mbipom, E. W. thesis, Univ. Edinburgh (1980).
14. Kurtz, R. D. & Garland, G. D. *Geophys. J.R. astr. Soc.* **45**, 321–348 (1976).

15. Hyndman, R. D. & Hyndman, D. W. *Earth planet. Sci. Lett.* **4**, 427–432 (1968).
16. Caner, B. J. *Geomagn. Geoelec.* **22**, 113–129 (1970).
17. Dowling, F. L. J. *geophys. Res.* **75**, 2683–2698 (1970).
18. Berdichevsky, M. N., Vanyan, L. L., Feldman, I. S. & Porstendorfer, G. *Beitr. Geophys.* **81**, 187–196 (1972).
19. Edwards, R. N. & Greenhouse, J. P. *Science* **188**, 726–728 (1975).
20. Van Ziji, J. S. V. *The Earth's Crust*, 470–500 (AGU Monograph No. 20, 1977).
21. Nekut, A., Connerney, J. E. P. & Kuckes, A. J. *Geophys. Res. Lett.* **4**, 239–242 (1977).
22. Sternberg, B. K. J. *geophys. Res.* **84**, 212–218 (1979).
23. Stanley, W. D., Boehl, J. E., Bostick, F. M. & Smith, H. W. J. *geophys. Res.* **82**, 2501–2514 (1977).
24. Hermance, J. F. & Pedersen, J. *Geophys. J.R. astr. Soc.* (in the press).
25. Duba, A., Piwinski, A. J., Santor, M. & Weed, H. C. *Geophys. J.R. astr. Soc.* **53**, 583–597 (1978).
26. Oxburgh, E. R. et al. *EEC 2nd int. Seminar on Geothermal Energy* 149–152 (1980).
27. Burley, A. J. et al. *EEC 2nd int. Seminar on Geothermal Energy*, 29–33 (1980).
28. Duba, A. *Acta geod. geophys. Montan.* **11**, 485–495 (1976).
29. Shankland, T. J. & Waff, H. S. J. *geophys. Res.* **82**, 5409–5417 (1977).
30. Wyllie, P. J. *Structure and Physical Properties of the Earth's Crust*, 279–301 (AGU Monograph No. 14, 1971).
31. Zbacki, M. D. thesis, Stanford Univ. (1975).
32. Lebedev, E. B. & Khitarov, N. J. *Geokhimiya* **3**, 195–201 (1964).
33. Law, L. K. & Riddihough, R. P. *Can. J. Earth Sci.* **8**, 1094–1106 (1971).
34. Gough, D. I. *Phys. Earth planet. Inter.* **7**, 379–388 (1973).
35. Garland, G. D. *Phys. Earth planet. Inter.* **30**, 220–230 (1975).
36. Phillips, W. E. A., Stillman, C. J. & Murphy, T. J. *geol. Soc. Lond.* **132**, 579–609 (1976).

Parental magma to the Bushveld Complex

G. Davies*, R. Grant Cawthorn*, J. M. Barton Jr† & Merryl Morton*

*Geology Department, University of the Witwatersrand, 1 Jan Smuts Avenue, Johannesburg 2001, South Africa

†Bernard Price Institute of Geophysical Research, University of the Witwatersrand, 1 Jan Smuts Avenue, Johannesburg 2001, South Africa

The Bushveld Complex of South Africa is a large layered intrusive suite of cumulate rocks for which the parental magma is not directly obtainable. However, we report here indirect evidence from a suite of fine-grained sills in the sediments below the Bushveld Complex. Mineralogical, geochemical and experimental petrological data are consistent with their being representative of the parental magma. They are siliceous picrites ($\text{SiO}_2 \sim 55\%$; $\text{MgO} \sim 12\%$), high in Cr, Ni, Rb and K, and with a high initial $\text{Sr}^{87}/\text{Sr}^{86}$ ratio, and hence represent a rather unusual magma composition. Crustal contamination may have been important in their evolution.

The layering and mineralogical variation in the Bushveld Complex, shown in Fig. 1, is accepted to be the result of fractional crystallization from a basic magma. However, the exact composition of this magma has been debated. Willemse¹ believed it to be similar to the voluminous metamorphosed gabbroic sills found in the sediments forming the floor to the Bushveld Complex; and suggested contamination of this liquid produced the noritic sills also found in the floor rocks. Typical analyses of these two suites of sills are given in Table 1.

Daly² and Wager and Brown³ studied fine-grained border facies which they believed might be original liquid compositions. Chemically, both are very similar to that proposed as the parental liquid to the Skaergaard Complex (see Table 1), which is surprising as the crystallization sequences and mineral compositions of the two complexes are quite different (Fig. 1).

Vermaak⁴ and Hamilton⁵ calculated a magma composition using modal proportions from the various zones based on an hypothesis of a new magma injection below the critical zone. Hamilton⁵ believed the MgO content of the parental magma exceeded 21% whereas Vermaak⁴ regarded it as having komatiitic affinities.

Beneath the Bushveld Complex to the east of Rustenburg, Transvaal, we have identified a suite of microproxenitic sills. These intrude the metamorphosed Silverton and Vermont shales and Magaliesberg quartzite of the Transvaal Supergroup⁶, and are found from 2 km below to the margin of the complex. They range in thickness from 3 to 10 m. Hopper and branching olivine and abundant bladed to feathery orthopy-

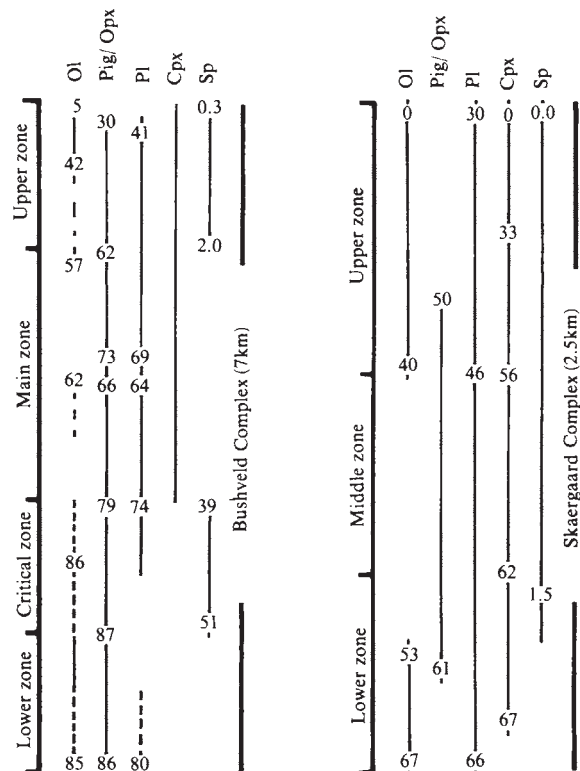


Fig. 1 Crystallization sequences and average mineral compositions in Bushveld and Skaergaard Complexes, from various sources^{3,9,16–20}. 100 Mg/(Mg + Fe) ratios for mafic minerals and An content of plagioclase are shown; dashed lines indicate sporadic appearance of cumulus phases. For the spinels, the V_2O_5 contents are shown, except for the minerals near the base of the Bushveld Complex, where numbers refer to the Cr_2O_3 contents.

roxene are present. The matrix is devitrified glass which contains occasional microlites of plagioclase and possibly clinopyroxene (see Fig. 2). These have been described in more detail elsewhere⁶. The crystallization of a small proportion of olivine, followed by a large amount of orthopyroxene, is consistent with the first-formed cumulates of the Bushveld Complex (Fig. 1) and so these sills could represent offshoots of the parental magma.

This suite of rocks is unusual in displaying high SiO_2 content (53–56%) and high MgO (9–13%) and has a Rb/Sr ratio of 0.15–0.20 (Table 1). It also has high K_2O , Ni and Cr.

We have studied several samples experimentally at 3 kbar pressure, one being from the chilled margin of a 3-m sill, occurring 600 m below the Bushveld Complex⁷. It has the second highest magnesium content of the samples analysed (analysis 1, Table 1) but its very fine-grained nature argues against any cumulus enrichment. Phase diagrams at 3 kbar pressure were constructed from our results (Fig. 3a, b); these are thought to be applicable to the crystallization of the Bushveld Complex. The composition of the chilled margin plots just within the olivine primary phase field in Fig. 3a and in the orthopyroxene primary phase volume in Fig. 3b.

The likely path it would follow during fractional crystallization is given on Fig. 3b by 1–A–B, involving olivine, orthopyroxene, orthopyroxene plus plagioclase, orthopyroxene plus plagioclase plus clinopyroxene. This crystallization sequence is very similar to that observed for the Bushveld Complex (see Fig. 1).

Trace element contents can also be compared with that expected of the parental magma to the Bushveld Complex. Hart and Davis⁸ found the partitioning of nickel between liquid and olivine to be dependent on the MgO content of the liquid. For a MgO content of 12.5% a value of $D_{\text{liq}}^{\text{Ni}}$ of 9 is obtained. The average NiO content⁹ of the lower zone olivine varies between 0.31 and 0.35%. Assuming that those are the first-formed

Table 1 Composition of micropyxenitic sills and some other proposed parental magmas

	1	2	3	4	5	6	7	8	9		10
SiO ₂	55.70	56.39	55.78	53.28	55.75	54.57	48.08	50.26	56.10	Q	3.18
TiO ₂	0.36	0.34	0.41	0.43	0.44	0.35	1.17	0.91	0.76	Or	6.07
Al ₂ O ₃	12.74	11.65	12.53	13.72	13.67	11.78	17.22	16.26	12.90	Ab	17.06
Fe ₂ O ₃	1.09	1.25	10.11*	10.64*	10.32*	10.04*	1.32	1.99	1.05	An	22.60
FeO	7.80	7.99					8.44	6.83	8.19	Di	9.55
MnO	0.09	0.19	0.19	0.13	0.16	0.16	0.16	0.13	0.18	Hy	39.28
MgO	12.44	13.05	9.71	9.22	9.34	11.36	8.62	6.32	10.12	Mt	1.58
CaO	6.96	6.42	6.50	7.95	7.30	6.25	11.38	9.70	6.38	Il	0.68
Na ₂ O	2.02	1.73	1.94	1.01	1.83	2.75	2.37	2.58	1.48		
K ₂ O	1.03	0.96	1.02	0.56	0.96	1.70	0.25	1.20	1.30		
Trace elements											
Ni	292	360	—	309	—	—	112	—	—		
Cr	970	1150	—	900	—	921	430	—	—		
Rb	37	35	—	21	—	54	10	—	—		
Sr	195	186	—	142	—	272	249	—	—		
Ba	439	461	—	—	—	723	—	—	—		
Zr	70	68	—	76	—	69	—	—	—		
Rb/Sr	0.19	0.19	—	0.15	—	0.20	0.04	—	—		

1, Proposed parental magma to the Bushveld Complex, fine-grained margin of a micropyxenitic sill.

2, Coarser central portion of the same sill as sample 1.

3, Mean of four analyses from a micropyxenitic sill from the eastern portion of the Bushveld Complex²².

4, Margin of micropyxenitic sill intruding Magaliesberg quartzite from which Rb–Sr isotope data were obtained.

5, Margin of micropyxenitic sill intruding the Silvertown shale ~2 km below the Bushveld Complex.

6, Central portion of micropyxenitic sill intruding the Vermont shale about 700 m below the Bushveld Complex.

7, Proposed parental magma to the Skaergaard Complex³, revised trace element data from Brooks and Nielsen²¹.

8, Typical metamorphosed gabbroic sill¹.

9, Typical noritic sill¹.

10, CIPW normative composition of sample 1.

* Total iron as Fe₂O₃.

minerals, the nickel content for the parental magma should be between 270 and 305 p.p.m., consistent with the data in Table 1.

Chromium partitioning between liquid and orthopyroxene is dependent on temperature and oxygen fugacity¹⁰. Assuming a value of between 1,350 and 1,300 °C for orthopyroxene crystallization temperature⁷ and a log *f*_{O₂} of –9, a partition coefficient of between 2.5 and 4 is obtained. The average Cr₂O₃ content⁹ of the lower zone orthopyroxene is 0.15%, which requires an original liquid with between 874 and 1,399 p.p.m. chromium. Again the data for these sills fall within this range.

The Rb/Sr ratio of the initial magma to the Bushveld Complex has been calculated from partition coefficients⁵ as being in the range 0.15–0.25: this is much higher than most basaltic liquids which vary between 0.01 and 0.05. The data in Table 1 show that these sills have this unusual value for the Rb/Sr ratio. The initial ⁸⁷Sr/⁸⁶Sr ratio, determined by Hamilton⁵, for the parental magma is 0.7056.



Fig. 2 Photomicrograph of bladed orthopyroxene embedded in very fine-grained matrix of devitrified glass. Hopper olivine crystals present in lower left of photograph. Field of view of the photomicrograph is 0.3 by 0.2 cm.

We have attempted to date an 8-m sill (Table 1, analysis 4) which has a moderate range in Rb/Sr ratios. The data do not define an isochron, but assuming an age⁵ of 2,095 Myr, the range in initial ⁸⁷Sr/⁸⁶Sr ratios is 0.7037–0.7098 with an average of 0.7067. Although more work will be required in this respect it does at this stage support the data of Hamilton⁵ and indicates a parental magma anomalously enriched in radiogenic Sr. This, when considered with the high concentrations of SiO₂, K₂O and Rb in such a magnesian magma, suggests that crustal contamination may have been a factor in the genesis of this suite of sills.

The new experimental data⁷ mean that previous ideas can now be reassessed. Figure 3 shows a typical gabbro composition (given in Table 1), believed¹ to be the parental magma. The two phase diagrams show the composition plots very close to the

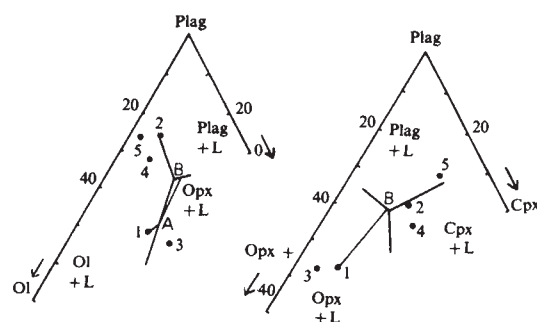


Fig. 3 Phase relationships at 3 kbar pressure from ref. 7 using the projection procedure of Irvine¹³. *a*, Clinopyroxene projection into the system olivine-plagioclase-quartz in cation proportions. *b*, Olivine projection into the system clinopyroxene-plagioclase-orthopyroxene + 4 quartz. The trend 1–A–B in the crystallization sequence is discussed in text. Sample numbers: 1, proposed Bushveld parental magma (analysis 1, Table 1); 2, typical metamorphosed gabbroic sill (analysis 8, Table 1); 3, typical noritic sill (analysis 9, Table 1); 4, proposed parental magma of the Bushveld Complex³; 5, proposed parental magma to the Skaergaard Complex^{3,21}.

olivine, clinopyroxene, plagioclase cotectic and as such will not crystallize the assemblages found for the Bushveld Complex (Fig. 1). Willemse's¹ other contention, that the norite rocks were derived from the gabbro liquid by contamination with siliceous sedimentary material, is questionable as the typical noritic composition (see Table 1) contains higher MgO than the gabbro, and so cannot possibly result from contamination of the latter.

Experimental studies at 1 atm pressure on Wager and Brown's³ sample gave a different crystallization sequence^{11,12} from that found in the Bushveld Complex, and Biggar¹² therefore rejected this as the parental magma. Daly's² sample was also studied by Tilley *et al.*¹¹: they were dubious about its being the parental magma to the Bushveld Complex as it also gave a different sequence to that observed in the Complex as a whole. Irvine's¹³ suggestion that possibly at higher pressures Wager and Brown's³ sample could produce the right crystallization sequence is not consistent with the phase diagrams in Fig. 3, where the early crystallization of clinopyroxene is implied. The calculations of Vermaak⁴ and Hamilton⁵ have been questioned by Von Gruenewaldt¹⁴ as the areal extent of the zones is a

matter for speculation. Obviously the results of such a calculation would be very dependent on the shape of the complex. If the complex is funnel-shaped as evidence cited by Button¹⁵ and Cameron⁹ suggests, then the effect of the lower olivine- and pyroxene-rich (MgO-rich) zones would be overestimated in these calculations. The models of Vermaak⁴ and Hamilton⁵ are also based on the assumption that there is a new magma influx below the critical zone. Cameron⁹ presented detailed mineral analyses across the lower zone-critical zone boundary and concluded there was no evidence for multiple magma injection at this level, at least in the eastern lobe of the Bushveld Complex.

None of the proposed parental magmas of Willemse, Daly, Wager and Brown, Vermaak or Hamilton seem very plausible, while the composition presented here seems in many respects to be consistent with the constraints required for it to be the parental magma to the Bushveld Complex.

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1. Willemse, J. *Proc. geol. Soc. S. Afr.* **62**, 21–80 (1959).
2. Daly, R. A. *Bull. geol. Soc. Am.* **39**, 703–768 (1928).
3. Wager, L. R. & Brown, G. M. *Layered Igneous Rocks* 588 (Oliver and Boyd, Edinburgh, 1968).
4. Vermaak, C. F. *Econ. Geol.* **71**, 1270–1298 (1976).
5. Hamilton, J. J. *Petrol.* **18**, 24–52 (1977).
6. Cawthorn, R. G., Davies, G., Clabey-Armstrong, A. & McCarthy, T. S. *Lithos* (in the press).
7. Cawthorn, R. G. *et al. Rep. Inst. Geol. Res. on the Bushveld Complex* 18, 27 (University of Pretoria, 1979).
8. Hart, S. R. & Davis, K. E. *Earth planet. Sci. Lett.* **40**, 203–219 (1978).
9. Cameron, E. N. *J. Petrol.* **19**, 437–462 (1978).

10. Schrieber, H. D. & Haskins, L. A. *Proc. 7th Lunar Sci. Conf.* 1221–1259 (1976).
11. Tilley, C. E., Yoder, H. S. Jr & Schairer, J. F. *Yb Carnegie Instn Wash.* **66**, 450–457 (1968).
12. Biggar, G. M. *Contr. Miner. Petrol.* **46**, 159–167 (1974).
13. Irvine, T. N. *Geol. Soc. S. Afr., Spec. Publ.* **1**, 441–476 (1970).
14. Von Gruenewaldt, G. *Can. Miner.* **17**, 233–256 (1979).
15. Button, A. *Trans. geol. Soc. S. Afr.* **79**, 3–12 (1976).
16. Von Gruenewaldt, G. *Trans. geol. Soc. S. Afr.* **76**, 207–227 (1973).
17. Molyneux, T. G. *Trans. geol. Soc. S. Afr.* **77**, 329–338 (1974).
18. Vincent, E. A. & Phillips, R. *Geochim. cosmochim. Acta* **6**, 1–26 (1954).
19. Lombard, B. V. *Trans. geol. Soc. S. Afr.* **59**, 59–74 (1956).
20. Molyneux, T. G. *Miner. Mag.* **38**, 863–871 (1972).
21. Brooks, C. K. & Nielsen, T. F. D. *Lithos* **11**, 1–14 (1978).
22. Sharpe, M. R. *Trans. geol. Soc. S. Afr.* **81**, 373–378 (1978).

Fossil carbon in coastal sediments

M. S. Baxter, M. J. Stenhouse & N. Drndarski

Chemistry Department, University of Glasgow,
Glasgow G12 8QQ, UK

Fossil carbon, as present in coal, oil and their derivatives, is free from radioactive ¹⁴C and its presence in even small concentrations within sedimentary materials can lead to anomalously old radiocarbon ages. For example, Erlenkeuser *et al.*¹ have noted artificially high ¹⁴C ages in the upper 20 cm of Baltic Sea sediments in correlation with enhanced heavy metal concentrations. Both findings are consistent with the growth of industrial activity in this location, and the radiocarbon age increase has therefore in the past been regarded as an approximate measure of the extent of pollution of sediment organics by industrial fossil fuels. The results presented here will indicate that the latter interpretation is not always valid and that in studies of coastal marine sediments the incorporation of natural fossil carbon in the samples can introduce considerable error.

Surface sediments from the Clyde Sea area in south-west Scotland were analysed and their radiocarbon data compared to those reported by other workers (ref. 2 and D. D. Harkness, personal communication) (Table 1). These sediments were brown muds of silt-clay composition and had carbon contents typically around 5% on a dry weight basis. In each case these surface deposits were known to have accumulated during the twentieth century, their radiocaesium and ²¹⁰Pb contents having been monitored³. From each sample, inorganic carbonaceous matter, normally comprising ~1.8% of sediment dry weight, was removed by hydrolysis in hot 15% HCl for 2 hours. It is the residual component—the acid insoluble organic fraction—which is being discussed here. Radiocarbon analysis was performed by either CO₂ gas proportional or C₆H₆ liquid scintillation techniques using conventional methodology^{4,5} and the

radiocarbon dates are reported in the normal way⁶, using the 5,568-year Libby half-life value. For the data presented in Table 1, the standard deviation associated with analysis is normally of minor significance relative to the age differences under discussion but in all cases is less than ±200 yr. The principal observation from the data is that surface ¹⁴C ages are extremely large relative to the idealized near-zero values which should apply. There is therefore a significant concentration of fossil carbon present. This in itself is scarcely surprising given that the Clyde marine system receives major inputs of both industrial and domestic organic wastes from the Glasgow conurbation and surrounding regions⁷. While there is some localized variability in ¹⁴C age distribution, the general ¹⁴C age trend shown in Table 1 can be reasonably interpreted as reflecting the corresponding degree of pollution exposure, with sites nearest industrial releases having oldest ¹⁴C ages. At the two extremes in the Table 1 data, ages of 1,595 yr (Loch Etive) and 10,490 yr

Table 1 Radiocarbon ages of surficial sediments

Sample	¹⁴ C age (yr)	Ref.
Loch Etive*	1,595	†
Loch Goil, core 1	2,320	This work
Loch Fyne, core 1	2,650	2
Loch Striven	3,255	2
Loch Goil, core 2	3,340	This work
Loch Goil, core 3	3,765	This work
Loch Fyne, core 2	4,405	2
Gare Loch	5,965	This work
Garroch Head†	4,120–10,490	2

*Loch Etive result included for comparison only—this site lies outside the Clyde area on the west coast of Scotland.

†Garroch Head, in the inner Firth of Clyde, is the disposal site for Glasgow's domestic/industrial solid waste. ¹⁴C ages decreased from 4,120 yr at the surface to 10,490 yr at 30 cm (ref. 2).

‡D. D. Harkness, personal communication.

(Garroch Head) correspond to fossil carbon concentrations (in the organic fraction) of ~18% and 73% respectively. At this stage it was tempting to postulate anthropogenic release of fossil carbon as the sole cause of the observed age anomalies.

The set of results which sheds most light on the issue, however, comes from a 3-m long sediment profile at a sampling site in the Arran Deep trench, a location in the outer Firth of Clyde which is considered relatively natural and far removed from organic waste releases. Here the sediment is very fine-grained brown to grey mud of total carbon content ~2%, approximately one third of which is in carbonate form. The latter fraction was removed by acid hydrolysis as before. The results surprisingly show that erroneously old ^{14}C dates are not confined to post-industrial horizons but occur throughout the profile (Fig. 1). That abnormally high ^{14}C ages are obtained even at depth shows clearly that the contaminant there is of natural origin and suggests that, in the recent sediments considered so far, a combination of both natural and industrial fossil carbon inputs is occurring.

Evidence that the deeper section of the Arran Deep core is pre-industrial and that its ^{14}C chronology is misleading comes first from palaeomagnetic marking of horizons which places the 1.75-m level at ~600 yr before present (R. Thomson and G. M. Turner, personal communication). In addition, the ^{14}C age profile is consistent with a pre-industrial period of constant sedimentation rate (between 170 and 260 cm), which by regression analysis of the data in that interval is assessed at $0.6 \pm 0.1 \text{ mm yr}^{-1}$ (coefficient of correlation, 0.99). This estimate is in good agreement with a previous assessment of 0.3 to 0.6 mm yr^{-1} for a neighbouring location². To a first approximation, the regression line drawn in Fig. 1 can therefore be regarded as the baseline representing the natural ^{14}C age profile at this site. Extrapolation to zero depth shows that the natural ^{14}C age for surficial deposits is ~3,000 yr, corresponding to a fossil carbon content of ~30%. The explanation of the high natural ages must, in our view, involve the natural erosion and

inwash of coal dust from the outcrops of coal seams in the Clyde coast and catchment. This postulate is supported by the result of attempts to totally dissolve deep sediment samples in strong acids. In all cases, a fine black suspension of particulates remains, the carbon content of the dried dust being 60 to 70%. The overall abundance of the coal dust is sufficient to produce the observed age anomalies but would not normally be isolated during conventional pretreatment techniques. Thus when an aliquot of sediment, from 250 cm depth, containing 68 mg of organic carbon was exhaustively treated with HF, the residual particulate material was found to contain 26 mg of organic carbon, corresponding to ~35% of the total acid-insoluble organic fraction. Furthermore, the sediment was shown by microscopic examination to be rich in Carboniferous spores and it therefore seems certain that the high ^{14}C ages in Clyde sediments primarily reflect natural coal erosion in the catchment, a process whose effect has not to our knowledge been reported elsewhere and which could induce major dating errors or be relevant to interpretation of dates from many other areas. Certainly, the magnitude of the effect, the chemical inertness and appearance of the contaminant and the presence of Carboniferous spores combine to rule out the possibility that the observed ^{14}C dilution is caused by soil and blanket peat erosion, a process frequently reported elsewhere^{1,8-11}, being induced by changes in catchment land use.

That the upper data points lie to the high-age side of the natural chronology (represented by the straight line) reflects anthropogenic disturbance of the sedimentation pattern. Thus input of fossil carbon by industry and waste dumping further ages the topmost layers and is allied to a much increased deposition rate. While the sampling frequency is insufficient for detailed analysis, it is possible that the uppermost sample is slightly younger through incorporation of artificial ^{14}C released mainly in the 1960s by nuclear weapon tests. In agreement with our interpretation of the ^{14}C profile, ^{210}Pb and radiocaesium analyses (J. Smith-Briggs, personal communication) have shown that the present sedimentation rate is ~20 mm yr⁻¹. The maximum ^{14}C age observed in surface layers (5,450 yr at 15 cm) corresponds to a minimum total fossil carbon contribution of ~50%. Thus at this site the natural fossil carbon contributes 30% and the industrial component 20%.

The results presented briefly here indicate the need to consider natural fossil carbon contamination as a potential and significant source of error in dating sediments. This natural component applies at all sediment depths, and, if constant, does not preclude estimation of deposition rates. In recent surficial samples, however, the natural input is added to and masked by anthropogenic releases. If long cores are not available, the two fossil components cannot be differentiated and considerable errors may arise in evaluating the data. Certainly, in the Clyde Sea area, radiocarbon dating of sediments is dominated by these large fossil carbon effects.

We thank the staff of the Clyde River Purification Board, the Natural Environment Research Council and the crew of the NERC research vessel John Murray for collection of sediment samples. Examination of pollen and spores in the sediment was performed by J. H. Dickson. We also thank D. Thomson and S. Holland for technical assistance.

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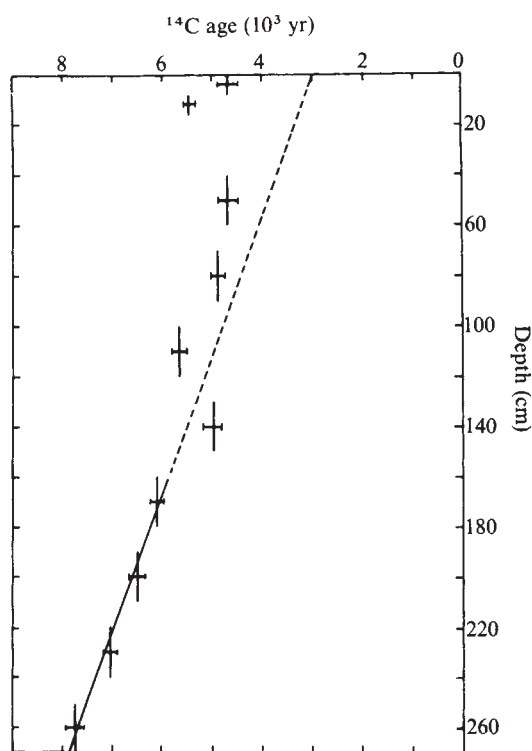


Fig. 1 Radiocarbon age/depth profile in Arran Deep sediments. Horizontal and vertical bars represent the $\pm 1\sigma$ dating errors and sample depth intervals respectively. The straight line is the best fit regression line through the lower data points.

1. Erlenkeuser, H., Suess, E. & Wilkomm, H. *Geochim. cosmochim. Acta* **38**, 823-842 (1974).
2. Baxter, M. S. & Harkness, D. D. *Proc. I.A.E.A. Symp. Isotope Ratios as Pollutant Source and Behaviour Indicators* 135-141 (1975).
3. Swan, D. S. thesis, Univ. Glasgow (1978).
4. Stenhouse, M. J. & Baxter, M. S. *Radiocarbon* **18**, 161-171 (1976).
5. Campbell, J. A. & Baxter, M. S. *Radiocarbon* **21**, 171-179 (1979).
6. Broecker, W. S. & Olson, E. A. *Radiocarbon* **3**, 176-204 (1961).
7. Natural Environment Research Council, *Clyde Study Group, Report, NERC publ. ser. C*, No. 11 (1974).
8. Pennington, W. *Freshwater Biol.* **3**, 363-382 (1973).
9. O'Sullivan, P. E., Oldfield, F. & Batterbee, R. W. in *Quaternary Plant Ecology* (eds Birks, H. J. B. & West, R. G.) 267-278 (Blackwell, Oxford, 1973).
10. Pennington, W. in *The Effect of Man on the Landscape: The Highland Zone* (res. Rep. Br. Council for Archaeology, 1975).
11. Dickson, J. H. *et al. Nature* **274**, 548-553 (1978).

A model of epicontinental reef growth

Peter J. Davies & J. F. Marshall

Bureau of Mineral Resources, Geology and Geophysics,
Canberra City ACT 2601, Australia

The growth of coral reefs has been a controversial subject ever since Darwin's¹ subsidence theory. Scientists have argued over the major factors affecting growth²⁻⁴, but most have agreed that reefs grow dominantly along their windward margins⁵⁻⁷, a characteristic displayed in the Permian⁸ and Devonian⁹ reefs of New Mexico and Western Australia. Recently, however, Adey¹⁰ has indicated that the growth characteristics of windward margins are energy related, such that different growth characteristics are to be expected under different energy regimes. The Great Barrier Reef is a high-energy epicontinental shelf reef system on which it has been suggested¹¹⁻¹³ that, in the prevailing conditions, windward growth has been subordinate to vertical and leeward growth. We present here results from drilling and monitoring studies demonstrating that reef growth in a transgressive epicontinental shelf environment is first vertical and then backwards, especially on the leeward margin, when the reef reaches the stillstand sea level position.

Our main body of data stems from extensive geological studies on and between the reefs of the Southern Great Barrier Reef. Four reefs have been studied in detail, but we report here only the results from One Tree Reef (Fig. 1) where comprehensive and integrated geological¹⁴⁻¹⁷, and biological studies¹⁸⁻²⁰ have been conducted for 10 yr. Previously reported geological studies on this reef have defined its major physiographical features, relating them to features of the Pleistocene substrate. Thus the present reef rims surmount elevations in the substrate while the present lagoon covers a depression in the Pleistocene surface¹⁶.

The Holocene section of the reef has been cored at six sites (Fig. 1), three near the southern, windward margin (1, 2 and 4), two on the leeward margin (3 and 5) and one on a patch reef in the lagoon (6). The facies variations encountered in four of these

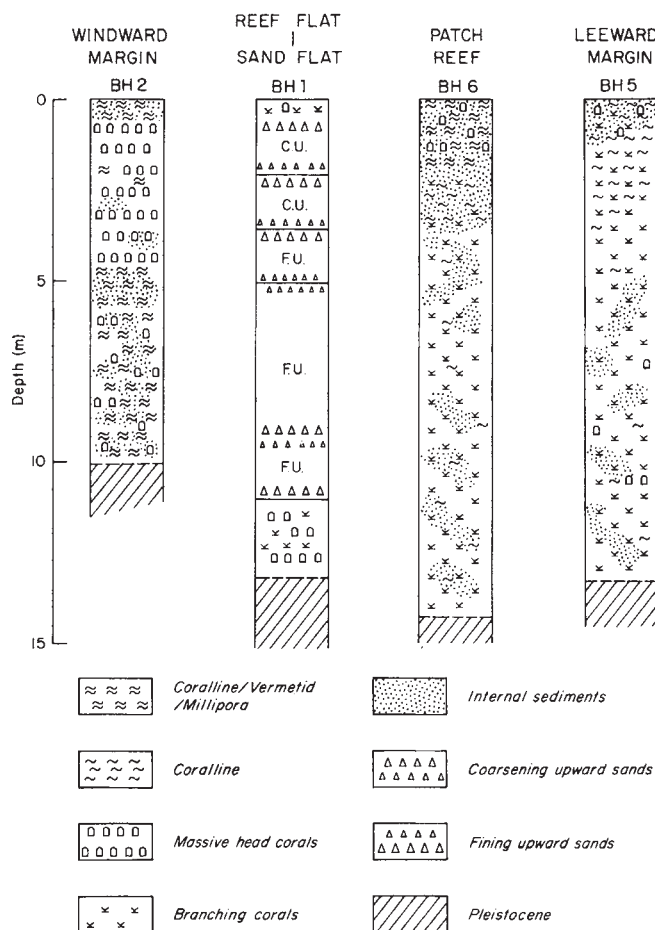


Fig. 2 Stratigraphic sections through Holocene at One Tree Reef, Southern Great Barrier Reef. Drill hole locations are shown on Fig. 1. % recoveries are BH1, 40%; BH2, 95%; BH5, 60%; BH6, 76%.

holes are shown in Fig. 2, from which the following conclusions are drawn.

Growth on the windward margin is dominated by head corals together with a coralline algae/*Millepora*/vermetid assemblage that forms a massive framework (hole 2). The latter assemblage especially dominates the lower section of the drill hole whereas at the surface it forms only thin crusts.

Sands, compositionally identical to and clearly part of the accreting sand wedge (Fig. 1), were encountered to a depth of 11 m in hole 1, drilled at the junction of the reef flat and sand wedge. The lower half of the sand sequences exhibits fining-upward rhythms indicative of episodic storm supply from the reef margin and representing the earliest, deeper subtidal stages of lagoon infill. The upper portions of the sand sequence show a series of coarsening-upward rhythms, representing very shallow subtidal to intertidal accretion. Identical rhythms are seen in vibrocores through the top 5 m of the accreting sand wedge (Fig. 1).

The leeward lagoonal patch reef (hole 6) is surprisingly dominated in the top part of the section by the head coral/coralline algae-*Millepora*-vermetid association, similar to that seen in the windward hole. Porosity is low due to multiple generations of internal sediment. Branching corals predominate throughout the rest of the section.

At leeward sites (hole 5), a much thinner coralline crust with subordinate head corals overlies a dominantly branching coral section forming an open framework.

Radiocarbon dating of corals from three of the boreholes (Fig. 3) shows (1) that growth on windward margins (hole 2) throughout much of the Holocene occurred at a rate of 3.2 m kyr⁻¹ except for a short burst in the top part of the section

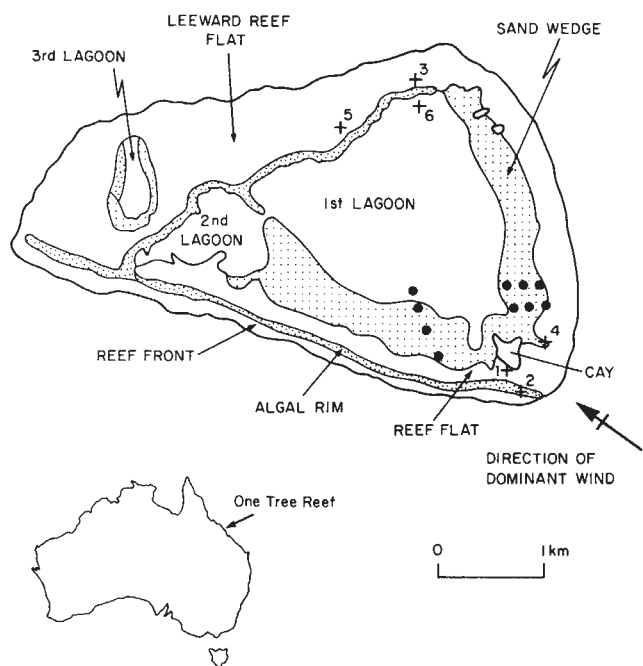


Fig. 1 One Tree Reef: physiographic features and sample sites. +, Drill sites; ●, sediment cores.

when the rate doubled; (2) that growth on the leeward margins (hole 3) throughout all but the bottom 1.5 m of section was nearly twice as fast as growth on the windward side; (3) that the growing reef reached sea level around 5,000 yr BP on the windward side compared with 1,000 yr BP on the leeward side; and (4) that in hole 1, the slow accretion rate of 1.7 m kyr^{-1} relates to the vertical growth of the accreting sand wedge and correlates well with independent estimates of lagoonal sand accumulation^{21,22}. Much of the sand section accumulated only after the windward margin reached sea level, suggesting that little material is shed during the vertical growth phase (Fig. 3).

Growth at the stillstand sea level has been studied by monitoring the relations between calcification and sedimentation. Net calcification estimates at One Tree Reef¹⁸⁻²⁰ show the windward perimeter zones (algal and coral flat) accreting at a rate of $4 \text{ kg m}^{-2} \text{ yr}^{-1}$. Measurements of water and sediment movement show daily losses²³ from the windward algal rim and reef flat of 0.6 and 1.8 kg respectively, this being to leeward, into the lagoon. Similar measurements on the leeward margin indicate differences in sediment transported on neap and spring tides. During neaps, there is a daily excess of 0.6 kg entering the lagoon from the leeward edge whereas during springs, the lagoon water is flushed quickly resulting in an excess of 2.9 kg transported out of the lagoon and over the leeward margin. A conservative estimate would indicate considerably more than 2,000 tonnes of CaCO_3 moving over the leeward margin every year. Seismic reflection profiling shows a wedge of sediment, interpreted as Holocene, up to 10-m thick extending 1 km away from the leeward margin (D. Searle, unpublished data).

We conclude that when a reef reaches sea level, growth is markedly influenced by the surface hydrological regime, resulting in lagoonal infill and leeward accretion.

Our data have implications for both growth concepts and reef management. In conditions of sea level rising at a rate greater than the rate of calcification, the dependence of growth on light will give rise to vertically growing structures. These will eventually be drowned if sea-level rise continues to outpace growth. However, if sea-level rise slows or reaches a stillstand position, vertical growth will continue, with little loss through shedding, until such time as the potential accretion exceeds that of sea-level rise. At such time as the hydrological regime imposes its own growth restrictions, accretion will dominate on the leeward margins and the reefs will grow to leeward. That this mechanism also applies to other parts of the Great Barrier Reef is shown by reports from the 1973 Royal Society Expedition to the area²⁴.

The total growth history of an epicontinental reef system such as the Great Barrier Reef is one of a series of growth phases alternating, during the Plio-Pleistocene at least, with prolonged periods of subaerial exposure. The record will, therefore, consist of a series of reefal and related sediments separated by

solution unconformities²⁵. If past growth approximates that which has occurred in the Holocene, then the facies patterns produced will resemble those defined in the Holocene, and will differ substantially from those described in the classically accepted models; for example, the windward and leeward directions are reversed. Such a postulate must have important palaeogeographic and mineral exploration significance. Implicit to our model is the understanding that the depth to the Pleistocene substrate determines the time at which the reef reaches sea level and, therefore, the length of time for which changes in the surface zone have occurred. It follows that some reefs may have reached sea level recently, exhibiting few characteristics of leeward accretion: such reefs will contrast with others where lagoons are infilled and where the leeward margin has prograded considerably. We designate reefs which have just reached sea level as juvenile, and those with considerable leeward progradation as senile. One Tree Reef, which is intermediate between senile and juvenile, is described as mature. All reefs in the Capricorn/Bunker Groups of the Southern Great Barrier Reef may be identified in one of these three classes, and we suggest that such a genetically based growth classification may have considerable managerial importance. For example, juvenile and senile reefs may react very differently to environmental perturbations, either natural or man-made.

Received 5 February; accepted 2 July 1980.

1. Darwin, C. *The Structure and Distribution of Coral Reefs* (Smith, Elder, London, 1842).
2. Murray, J. *Proc. R. Soc. Edinb.* **10**, 505-518 (1880).
3. Daly, R. A. *Proc. Am. Acad. Arts Sci.* **51**, 157-251 (1915).
4. Davis, W. M. *Am. geogr. Soc. Spec. Publ.* **9** (1928).
5. Holmes, A. *Principles of Physical Geology* (Nelson, Sunbury-on-Thames, 1965).
6. Thornbury, W. D. *Principles of Geomorphology* (Wiley, New York, 1969).
7. Maxwell, W. G. H. *Atlas of the Great Barrier Reef* (Elsevier, Amsterdam, 1968).
8. Newell, N. D. *et al.* *The Permian Reef Complex of the Guadalupe Mountains Region, Texas and New Mexico* (W. H. Freeman, San Francisco, 1953).
9. Playford, P. E. *Western Australia Geol. Surv. Rep.* **1** (1969).
10. Adey, W. H. *Science* **202**, 831-837 (1978).
11. Davies, P. J. *Proc. 3rd int. Coral Reef Symp.* **2**, 325-330 (1977).
12. Davies, P. J. *et al.* *Proc. 3rd int. Coral Reef Symp.* **2**, 331-338 (1979).
13. Stoddart, D. *et al.* *Proc. R. Soc. B284*, 149-159 (1978).
14. Davies, P. J., Radke, B. M. & Robison, C. R. *BMR J. Aust. Geol. Geophys.* **1**, 231-240 (1976).
15. Davies, P. J. & Kinsey, D. W. *Mar. Geol.* **24**, 1-11 (1977).
16. Harvey, H., Davies, P. J. & Marshall, J. F. *BMR J. Aust. Geol. Geophys.* **4**, 141-147 (1979).
17. Davies, P. J. & Marshall, J. F. *Search* **10**, 776-779 (1979).
18. Kinsey, D. W. *Proc. 1st int. Coral Reef Symp.* **13-32** (1972).
19. Kinsey, D. W. & Davies, P. J. in *Biogeochemical Cycling of Mineral-forming Elements* (eds Trudinger, P. & Swaine, D.) 131-162 (Elsevier, Amsterdam, 1979).
20. Kinsey, D. W. & Davies, P. J. *Limnol. Oceanogr.* **24**, 935-940 (1979).
21. Frankel, E. *Proc. 3rd int. Coral Reef Symp.* **2**, 201-208 (1977).
22. Davies, P. J. *Reef Growth: A Review* (Australian National University Press, in the press).
23. Davies, P. J. & West, B. (in preparation).
24. Orme, G. R., Flood, P. G. & Sargent, G. *Proc. R. Soc. A291*, 85-100 (1978).
25. Davies, P. J. *Proc. 2nd int. Coral Reef Symp.* **2**, 573-578 (1974).

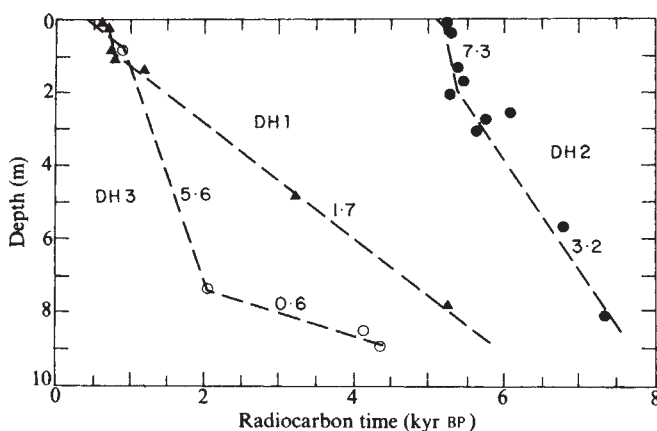


Fig. 3 Rates of reef accretion (m kyr^{-1}) displayed in three boreholes at One Tree Reef. Note that accretion in hole 1 starts only after the perimeter margin (hole 2) has reached sea level around 5,000 yr BP. Only corals in growth position have been dated.

Oxidative changes to nitrate and boron in marine pore waters

Kent A. Fanning & Valentine I. Maynard-Hensley

Department of Marine Science, University of South Florida, St Petersburg, Florida 33701

Reported changes in the composition of marine pore water on contact with air¹⁻⁴ led us to investigate whether air-exposure of marine sediment might alter its interstitial concentrations of nitrate and boron. Nitrate should be a good indicator of changes in the oxidation state of marine sediments^{5,6}, and interstitial boron should reflect changes in the reactivity of sedimentary components because it bonds to clays^{7,8} and sediments in a temperature-sensitive manner. We report here that interstitial nitrate concentrations can increase considerably during air exposure of marine sediment and that interstitial boron can be scavenged from some reducing sediment. Sedimentary boron scavenging may affect oceanic boron, but the process may be influenced by interstitial components that prevent the uptake of boron onto freshly formed hydrous ferric oxides.

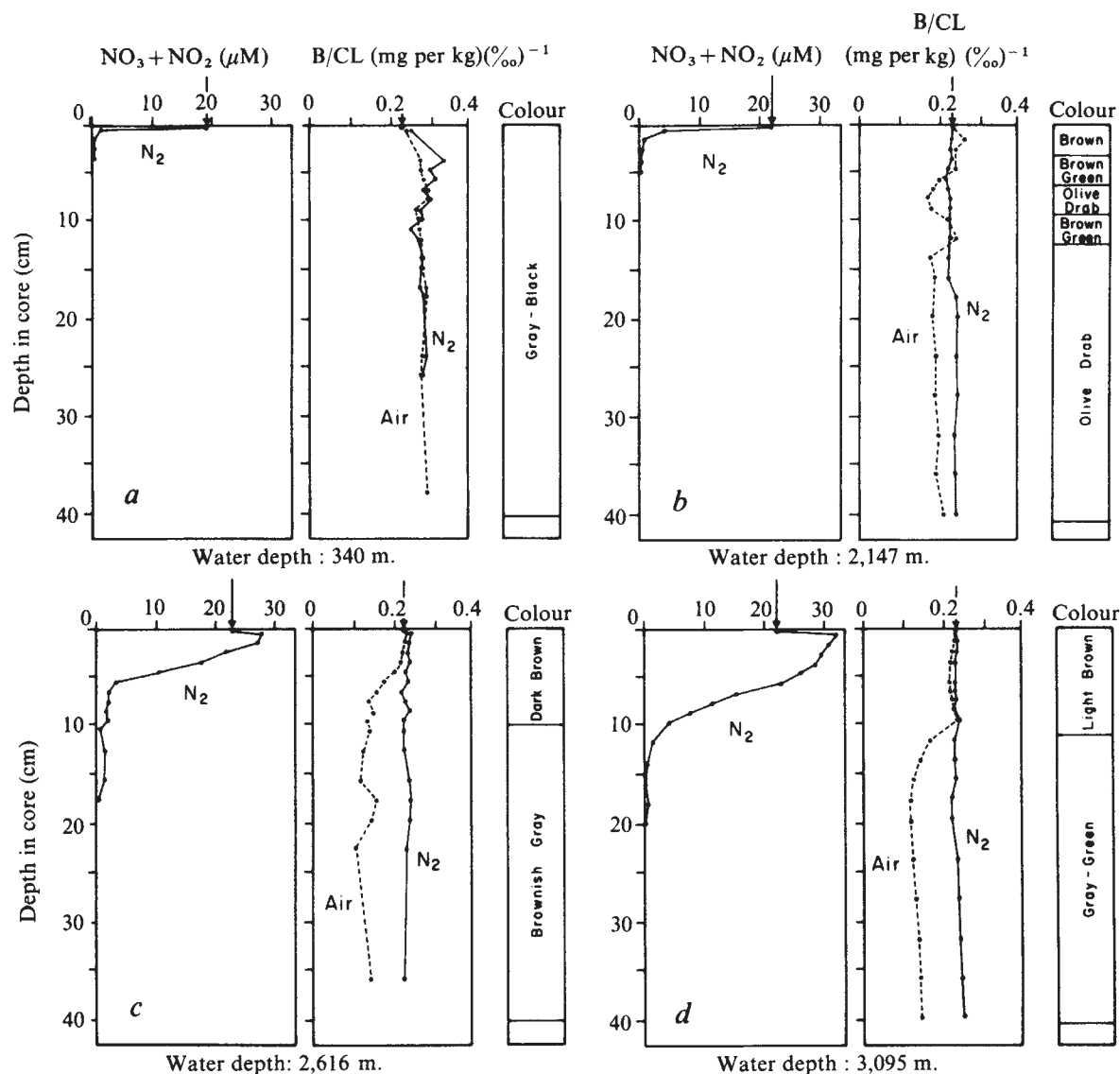


Fig. 1 Air-squeezed (····) and nitrogen-squeezed (—) profiles of interstitial ratios of boron to chlorinity and interstitial concentrations of nitrate + nitrite in sediments from the delta of the Magdalena River. The arrows indicate values of near-bottom seawater above each core. Colour patterns in the sediment are also shown. A Benthos Model 2171 gravity corer was deployed from the RV Gyre out of Texas A & M University. To minimize disturbance during coring, no cutter, catcher, or barrel was used, and the lowering speed on the winch was less than 20 m min^{-1} . *a*, 340 m water depth; *b*, 2,147 m water depth; *c*, 2,616 m water depth; *d*, 3,095 m water depth.

In August 1977, four 6.5-cm diameter gravity cores were taken from different water depths on the Magdalena River delta near Barranquilla, Columbia (Table 1). Each core was immediately sealed and transferred to a room kept at around the same temperature as that where the core was obtained: 17°C for the 340-m core and 4°C for the cores from deeper water (Table 1). Each core was opened and cut into 1-cm slices inside a nitrogen-filled plastic bag at positive pressure. Half of each slice was squeezed to remove its pore water while still inside the bag; the other half was exposed to air for 1–2 h and then squeezed to extract its pore water in oxygenating conditions. For each core, the processing temperature was the same as its storage temperature.

Air-squeezed and nitrogen-squeezed pore waters were analysed for dissolved boron¹⁰, for dissolved nitrate and nitrite¹¹, and for chlorinity using AgNO_3 with dichlorofluorescein as endpoint indicator. Air-squeezed and nitrogen-squeezed profiles for each core were compared to each other and to the core's colour pattern (Fig. 1a–d).

Air-exposure of Magdalena sediment produced strong enrichments in interstitial nitrate + nitrite (Table 2). The increases were usually confined to the upper parts of the cores, and the largest were in the upper 4 cm of the core from 2,616 m

of water. The zone seemed to be reasonably oxidizing because of its high initial concentrations of nitrate and because of its dark brown colour¹². Air exposure did not produce large enrichments in sediment which was so reducing that nitrate or nitrite was originally absent. (Possibly the microorganisms whose metabolism might be stimulated by air are not likely to be important in such sediment.) Thus conclusions based on nitrate data from air-exposed sediment may be suspect, even for deeper-water deposits thought to be oxidizing. Also, atmospheric contact may stimulate nitrate production in tidally exposed sediment.

Figure 1 shows only nitrogen-squeezed profiles of nitrate + nitrite for comparison to the profiles of air-squeezed and nitrogen-squeezed interstitial boron. For most of the Magdalena cores, interstitial nitrite concentrations were small (around $0.2 \mu\text{M}$), and we will consider only nitrate here.

Figure 1 shows the boron-chlorinity ratios (B/CL) in the pore-water samples. The conservative nature of oceanic boron means that all results may be compared with the typical value of the ratio: $0.23 \text{ (mg B per kg)} (\text{‰})^{-1}$ (see refs 13, 14).

The Magdalena profiles show that interstitial boron can be strongly scavenged from marine pore fluids when atmospheric oxygen penetrates the exposed sediment. In two cores from deeper water (Fig. 1c, d), interstitial boron-chlorinity ratios

were reduced by as much as 50%, indicating a consumption of 210 μM borate or 2.2 mg B per kg of pore water. In the third deeper-water core (Fig. 1b), the loss was less. In the shallowest core (340 m, Fig. 1a) the ratios were slightly higher than normal, probably because the room in which the 340 m core was processed was a little too warm at 17 °C. Seawater temperature at 340 m was 12 °C, and a 5 °C increase should cause interstitial boron concentrations to rise detectably⁹.

No zone showing strong scavenging of boron contained much nitrate. Air-sensitivity of interstitial boron was most likely when the sediments were so reduced that interstitial nitrate was 5 μM or less. When nitrate concentrations were higher, a solid phase capable of removing boron was apparently absent or too oxidized to function.

Actual scavenging phases in reducing zones could have been freshly precipitated forms of Fe(III) which were created when atmospheric oxygen reacted with Fe(II) that was either dissolved in pore water or attached to particle surfaces¹⁻³. In cores from water deeper than 2,000 m (Fig. 1b-d), zones of sediment that showed no scavenging of interstitial boron had brownish colours, suggesting that Fe(III) dominated over Fe(II) in the solid phases¹². However, intensity and distribution of boron scavenging varied considerably in the four Magdalena cores. Organic matter in the sediment may influence the degree of scavenging because of the affinity of borate for carbohydrates and other organic compounds with alcohol groups^{15,16}.

The uptake of oceanic boron by iron compounds was studied by using two laboratory simulations of the action of sedimentary ferrous iron during air exposure at 4 °C. In both, 500 ml of pelagic seawater was enriched with 'scavengeable' nutrients until the initial concentrations were 200 μM silica and 20 μM phosphate—values often found in reducing marine pore waters³. Initial boron concentrations were 406 μM (4.29 mg B per kg seawater) in the first experiment and 418 μM (4.42 mg B per kg seawater) in the second. To inhibit the oxidation of Fe(II), the enriched batches of seawater were bubbled with nitrogen for 2 h to remove dissolved oxygen and then acidified to pH 3 with concentrated H_2SO_4 . $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ was added to each flask to make its acidified seawater 13,000 μM in dissolved Fe(II) so that there was initially a 20:1 molar ratio of ferrous iron to total scavengeable solutes (silica + phosphate + boron). In the second experiment, the seawater was also made 20,000 μM in mannitol to see if a large amount of boron-complexing organic matter altered the scavenging of boron. Then, over a 2-h period, oxidative scavenging was induced by

Table 1 Data on cores from the Magdalena Delta

Core no.	Water depth (m)	Position
77-G-8, G.C. 2	340	11°08.4' N, 74°54.8' W
77-G-8, G.C. 8	2147	11°23.5' N, 75°25.3' W
77-G-8, G.C. 7	2616	11°39.5' N, 75°33.5' W
77-G-8, G.C. 6	3095	11°49.5' N, 75°38.1' W

adding concentrated NH_4OH dropwise while stirring and bubbling air into the flasks. As the first drops of NH_4OH entered the seawater, a bluish precipitate formed which eventually turned red-brown as air bubbles mixed with it. Towards the end of each experiment, the drops of NH_4OH produced more red-brown precipitate directly with no bluish intermediate. Addition of NH_4OH was stopped when the pH passed 8.5 and dissolved iron was less than 3% of its initial value. The reaction flasks were polythene.

Final aliquots were removed from the two batches of seawater, filtered through 0.4 μm Nuclepore membranes, and analysed. The dissolved silica and phosphate initially present in both experiments were at least 98% removed. In the first experiment, the boron-chlorinity ratio was 12% lower after the precipitation of Fe(III) hydroxide was finished. However, in the second experiment, the added manitol apparently restricted the scavenging of boron because the final boron-chlorinity ratio was only 2% less than the initial ratio.

There are two curious features of the Magdalena data (Fig. 1): (1) interstitial nitrate was absent or nearly absent from any sediment in which a sizeable air-induced uptake of interstitial boron occurred, and yet (2) the absence of nitrate did not guarantee an air-induced uptake of boron, for example in most of the 340 m core and in parts of the upper 12 cm of the 2,147 m core.

The laboratory simulations provide partial explanations. Freshly oxidized and precipitated Fe(III) oxide did scavenge boron from seawater along with silica and phosphate. Marine sedimentary Fe(II) that can form fresh Fe(III) oxides is apparently not present until interstitial nitrate has been consumed by reduction reactions¹⁷. Therefore, air-induced

Table 2 A comparison of concentrations of nitrate + nitrite in nitrogen-squeezed and air-squeezed pore waters from sediment cores from the Magdalena River delta. Concentration units are μmol per litre of pore water

Depth in core (cm)	Core G.C. 2 (water depth, 340 m)			Core G.C. 8 (water depth, 2,147 m)			Core G.C. 7 (water depth, 2,616 m)			Core G.C. 6 (water depth, 3,095 m)		
	N ₂	Air	Δ	N ₂	Air	Δ	N ₂	Air	Δ	N ₂	Air	Δ
0-1	1.1	8.2	7.1	4.3	—	—	27.3	72.0	44.7	31.6	30.0	-1.6
1-2	0	2.0	2.0	1.0	20.4	19.4	26.8	45.4	18.6	30.5	31.1	0.6
2-3	0	1.2	1.2	0.8	11.5	10.7	21.4	31.9	10.5	29.3	32.0	2.7
3-4	0	0	0	0.4	—	—	17.2	23.7	6.5	28.2	29.7	1.5
4-5	0	0	0	0	2.5	2.5	10.3	9.5	-0.8	25.9	31.4	5.5
5-6	0	0	0	0	0	0	2.8	2.1	-0.7	22.5	26.4	3.9
6-7	0	0	0	0	0	0	2.0	2.7	0.7	15.3	16.3	1.0
7-8	0	0	0	0	0	0	1.7	1.6	-0.1	11.3	12.7	1.4
8-9	0	0	0	0	0	0	1.3	0.7	-0.6	7.4	9.3	1.9
9-10	0	0	0	0	0	0	1.4	1.5	0.1	4.1	4.3	0.2
11-12	0	0	0	0	0	0	0.4	2.7	2.3	1.4	2.6	1.2
13-14	0	0	0	0	0	0	1.2	2.7	1.5	0.5	1.9	1.4
15-16	0	0	0	0	0	0	1.0	1.3	0.3	0	0.8	0.8
17-18	—	0	—	0	0	0	0	1.5	1.5	0.5	0.5	0
19-20	—	0	—	0	0	0	0	0.7	0.7	0.2	0.5	0.3
23-24	—	0	—	0	0	0	0	0.6	0.6	0	0.3	0.3
27-28	—	0	—	0	0	0	0	0.4	0.4	0	0	0
31-32	—	0	—	0	0	0	0	0.7	0.7	0	0.4	0.4
35-36	—	0	—	0	0	0	0	0.6	0.6	0	0	0
39-49	—	0	—	0	0	0	0	0.3	0.3	0	0	0

boron scavenging and nitrate could be mutually exclusive in sediments. However, our experiments did not display the extensive boron losses found in much of the deeper Magdalena sediment. Perhaps insufficient Fe(II) was used compared with that available in sediments, or the surface area of sedimentary particles may be important. But the lack of boron scavenging in some reducing sediments is more puzzling. The simulations suggest that interstitial organic matter may have been responsible. Although mannitol is not a major organic compound in sediments, it has been reported for marine cores¹⁸, and we found that it hampered the scavenging of boron by Fe(III) oxides. It probably did not drastically alter the surface properties of the Fe(III)-oxide precipitates because silica and phosphate were scavenged with or without mannitol. Therefore, the mannitol may have complexed the boron so that it was not scavenged as

effectively. Similar 'protective' complexation between boron and sedimentary organic compounds may occur in natural reducing sediments. In fact, a weak response of interstitial boron to air exposure may be an effective indicator of the presence of such compounds in a sediment.

Marine sediment can encounter elemental oxygen in two forms: an atmospheric gas or an oceanic solute. Our results suggest that this encounter could stimulate reactions which become important parts of the cycles of nitrate and boron in the sea.

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1. Bray, J. T., Bricker, O. P. & Troup, B. N. *Science* **180**, 1362-1364 (1973).
2. Troup, B. N., Bricker, O. P. & Bray, J. T. *Nature* **249**, 237-239 (1974).
3. Loder, T. C., Lyons, W. B., Murray, S. & McGuinness, H. D. *Nature* **273**, 373-374 (1978).
4. Lyons, W. B., Gaudette, H. E. & Smith, G. M. *Nature* **277**, 48-49 (1979).
5. Pamatmat, M. M. *Int. Rev. Hydrobiol.* **58**, 345-368 (1973).
6. Bender, M. L., Fanning, K. A., Froelich, P. N., Heath, G. R. & Maynard, V. *Science* **198**, 605-609 (1977).
7. Lerman, A. *Sedimentology* **6**, 267-286 (1966).
8. Couch, E. L. & Grimm, R. E. *Clays Clay Miner.* **16**, 249-256 (1968).
9. Sayles, F. L., Manheim, F. T. & Waterman, L. S. *Init. Rep. DSDP Leg 20* (1973).
10. Uppström, L. R. *Analyt. chim. acta* **43**, 475-486 (1968).

11. Armstrong, F. A. J., Stearns, C. R. & Strickland, J. D. H. *Deep-Sea Res.* **14**, 381-389 (1967).
12. Sverdrup, H. U., Johnson, M. W. & Fleming, R. H. *The Oceans, Their Physics, Chemistry, and General Biology*, 967-969 (Prentice-Hall, Englewood Cliffs, 1942).
13. Uppström, L. R. *Deep-Sea Res.* **21**, 161-162 (1974).
14. Fanning, K. A. & Maynard, V. *Neth. J. Sea Res.* **12**, 345-354 (1978).
15. Braman, R. S. in *Treatise on Analytical Chemistry, Part II* Vol. 10 (eds Kolthoff, I. M. & Elving, P. J.) 1-101 (Wiley, New York, 1978).
16. Gast, J. A. & Thompson, T. G. *Analyt. Chem.* **30**, 1549-1551 (1958).
17. Froelich, P. N. et al. *Geochim. cosmochim. Acta* **43**, 1075-1090 (1979).
18. Modzeleski, J. E., Laurie, W. A. & Nagy, B. *Geochim. cosmochim. Acta* **35**, 825-838 (1971).

Records of *Cooksonia*-type sporangia from late Wenlock strata in Ireland

D. Edwards

Department of Plant Science, University College, Cardiff CF1 1XL, UK

J. Feehan

Department of Geology, Trinity College, Dublin 2, Ireland

Hitherto the earliest record of a fossil flora containing *Cooksonia* type sporangia was from a mid-Ludlow (Silurian) locality in Wales¹. The compression fossils illustrated here (Figs 1-4) come from strata assigned to the highest graptolite zone of the Wenlock Series characterized by *Monograptus ludensis*. They were collected (by J. F.) in the Devilsbit Mountain district of County Tipperary, Ireland. But this is not just a record of the first appearance of a genus which extends to the end of the Lower Devonian, because *Cooksonia* has generally been accepted as a vascular plant². Its occurrence at the top of the Wenlock therefore seems to extend the history of vascular plants back to the middle of the Silurian and to indicate that higher plants colonized land surfaces at least 415 Myr ago.

The very fragmentary plant fossils are scattered among numerous irregular patches of coalified material, the rock matrix being a slightly micaceous buff siltstone interlaminated with a grey-green mudstone from which fossils are absent. In marked contrast to later Ludlow records, sterile branching axes of *Hostinella*-type are rare and far outnumbered by the fertile specimens. One of the latter, which exhibits dichotomous branching, is shown in Fig. 1. The sporangia are tangentially extended and oval to irregular in outline. They most closely resemble *Cooksonia pertoni* Lang, but are considerably smaller. We have illustrated the remaining specimens to show some of the range in sporangial morphology, which indicates that more than one morphospecies is represented. As they are subtended by very short lengths of unbranched axis, they do not strictly conform to the generic diagnosis of *Cooksonia*, although one of

them (Fig. 4) is similar to *C. hemisphaerica* Lang in sporangial shape.

However, because we have not yet seen tracheids on cellulose acetate film pulls taken from these Irish compression fossils, we cannot be absolutely certain that they were vascular plants. Had they been found in later, for example, Downton (Pridoli) or



Fig. 1 Forking axis with two terminal sporangia ($\times 27$).



Fig. 2 Terminal sporangium ($\times 25$).

Gedinne sediments, such fossils would undoubtedly be assigned to *Cooksonia* and be accepted as vascular plants. In view of their great stratigraphical age and their relevance to such highly controversial issues as the ancestry and origin of vascular plants, the possibility that the Irish fossils had identical morphology to *Cooksonia* but lacked conducting tissues cannot be ignored. Pratt *et al.*³ described such a level of organization as rhyniophytoid. Sterile branching axes lacking vascular tissues and thought to belong to erect plants have been reported from early Silurian deposits in the United States⁴. The authors of that report suggested that the plants were "representatives of primitively emergent vegetation" (page D71) and "incipient vascular plants" (page D72). In a different physiological approach, Raven⁵ argued that the alga-vascular plant intermediate would require a plant body of some size in order to release its meiospores into air currents and that such spores with sporopollenin-impregnated walls would be produced at the apices of parenchymatous axes. Similarly Smith⁶ raised the possibility that the parent plants of the simple, smooth-walled spores with triradiate marks which he has isolated from Wenlock strata in County Mayo were non-vascular aquatics (? presumably with emergent sporangia).



Fig. 3 Reniform terminal sporangium ($\times 25$).

Thus practising the caution advised by Banks⁷, we can only conclude that the Irish flora certainly contains the earliest known erect fertile land plants and perhaps the earliest fertile vascular plants.

That they are the earliest *Cooksonia*-type sporangia is substantiated by faunal evidence. The necessity for accurate independent stratigraphical dating for any hypotheses relating to the phylogeny of vascular plants, in particular their ancestry and early evolution, has been emphasized elsewhere¹.

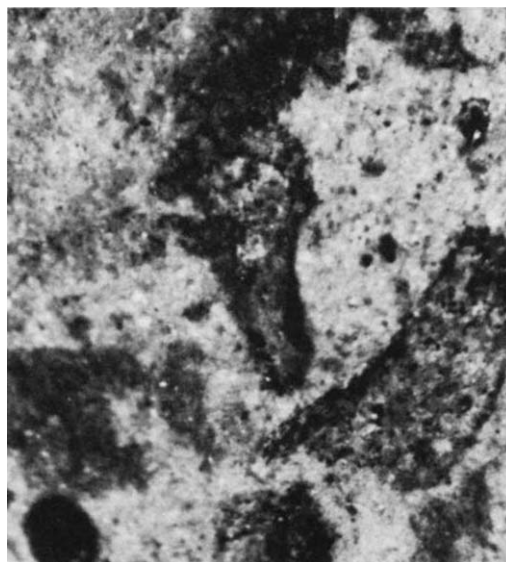


Fig. 4 Globose terminal sporangium ($\times 23$).

The rocks of the Devilsbit Mountain area constitute the northeastern end of an extensive inlier of Silurian rocks that extends south-westwards to include Silvermines and Slieve Phelim. The succession, the overall thickness of which is probably in excess of 2,500 m, comprises a monotonous succession of turbiditic sandstones, laminated siltstones and mudstones, with very rare microconglomerates and volcanic rocks. The siltstones are frequently fossiliferous, yielding a varied fauna of *Cyrtograptus ludgreni* Zone age over much of the inlier⁸⁻¹¹. To the south-east of Moneygall, near the northeastern end of the inlier, a small area of rocks of *Monograptus ludensis* Zone age is preserved in the core of a syncline in the vicinity of Cloncannon townland. These rocks are characterized by a fauna that includes *M. ludensis* Murchison, which is much the most abundant species present, and *M. auctus* Rickards together with nautiloids, phyllocarids, a small crinoid, occasional small brachiopods and epifaunal bivalves. The plant-bearing lithologies occur near the top of Borrisnoe Mountain, 1.7 km south-east of Moneygall (Grid reference 18 S 052782), near the southeastern edge of the Cloncannon syncline, and towards the base of the *ludensis* sequence, which attains a thickness of around 900 m. The age of the rocks in which the sporangia occur is confirmed by the presence of the two diagnostic graptolite species (identified by Dr R. B. Rickards) both above and below the plant fossils at the locality. Hence these Irish macroplants predate the lower *Baragwanathia* flora of Victoria, Australia even if the early Ludlow age is confirmed¹².

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1. Edwards, D., Bassett, M. G. & Rogerson, E. C. W. *Lethaia* **12**, 313-324 (1979).
2. Banks, H. P. *Bioscience* **25**, 730-737 (1975).
3. Pratt, L. M., Phillips, T. M. & Dennison, J. M. *Rev. Palaeobot. Palynol.* **25**, 121-149 (1978).
4. Schopf, J. M., Mencher, E., Boucot, A. J. & Andrews, H. N. *U.S. Geol. Surv. Prof. Pap.* **550-D**, D69-D75 (1966).
5. Raven, J. A. *Adv. bot. Res.* **5**, 153-219 (1977).
6. Smith, D. G. *Geol. Mag.* **112**, 411-414 (1975).
7. Banks, H. P. *Rev. Palaeobot. Palynol.* **20**, 13-25 (1975).
8. Cope, R. N. *Geol. Mag.* **91**, 319-324 (1954).
9. Cope, R. N. *Proc. Roy. Irish Acad.* **60B**, 217-242 (1959).
10. Palmer, D. *Sci. Proc. R. Dubl. Soc.* **A3**, 335-342 (1970).
11. Doran, R. J. P. *Proc. R. Irish Acad.* **74B**, 193-202 (1974).
12. Garratt, M. J. *Alcheringa* **2**, 217-224 (1979).

Perceptual learning specific for orientation and spatial frequency

Adriana Fiorentini & Nicoletta Berardi

Istituto di Neurofisiologia del Consiglio Nazionale delle Ricerche, Pisa, Italy

Several examples of 'perceptual learning' (improvement of some perceptual task with practice) have been reported¹⁻⁴. These studies are of great interest for neurological research because they demonstrate plasticity of the nervous system. Even for apparently basic perceptual tasks, such as visual acuity or vernier acuity^{1,4}, practice can facilitate a neural change which enhances performance. One question in this field is where does this learning occur? Indications about the possible neural site of a learning process may be derived from its specificity for some particular stimulus parameters. For instance, there is a hint that learning in global stereopsis may occur at a stage where visual information is processed by mechanisms selectively sensitive to different stimulus orientations³. We report here an experiment on perceptual learning in the discrimination of gratings of different waveform. Our findings show that learning is specific for both the orientation and the spatial frequency of the practice stimulus.

The aim of the experiment was to evaluate the effects of training on the ability to discriminate gratings of different luminance distributions, to determine whether these effects were retained over time and to ascertain whether the effects would transfer to gratings of different orientations or different spatial frequencies.

The patterns to be discriminated (Fig. 1) were two complex gratings consisting of the sum of two sinusoids of spatial frequency f and $3f$, and contrast 40 and 13%, respectively (≥ 1 log unit above threshold). The two gratings differed only in the relative spatial phase of the two components. In grating *a* the two sinusoidal components had spatial phase 0° , like the first and third harmonic of a square wave. In grating *b* the spatial phase of the third harmonic was shifted by 90° . The luminance profiles of the two gratings are shown in the inset of Fig. 2.

The gratings were generated on a circular oscilloscope screen (Tektronix 503, 12-cm diameter) by a PDP11/10 computer at the rate of 2 ms per frame and viewed binocularly, with natural pupils. A black fixation dot of 3 mm diameter was superimposed at the centre of the screen. The average luminance of the screen (10 cd m^{-2}) was kept constant during and between grating presentations.

Discrimination of pairs of gratings of the same orientation, contrast and spatial frequency and with the luminance profiles shown in Fig. 2 was assessed with a two-alternative temporal forced-choice procedure. The gratings were presented successively for 16 ms (8 frames) each, with an interval of 500 ms. The order of presentation was varied quasi-randomly from trial to trial. Subjects reported after each trial in which of the two time intervals (indicated by a sound) grating *a* had been presented, and were informed whether their response was correct.

Two experienced and four naive subjects participated in the experiments. All subjects took part in three experimental sessions. In the initial 50–70 trials of each session, the gratings to be discriminated were vertical and viewed from 1.2 m, so that their fundamental spatial frequency was 1 cycle per deg. In the subsequent 80 trials, the gratings were either viewed from the same distance, but orientated horizontally (first experimental session), or orientated vertically and viewed from 60 cm (fundamental spatial frequency 0.5 c/deg, second session) or from 2.4 m (fundamental spatial frequency 2 c/deg, third session).

To evaluate changes in discrimination performance with training, the responses of each subject were grouped in

subsequent blocks of 10 trials each. The corresponding blocks of trials of the six subjects were then pooled and the percentage of correct responses evaluated.

The results are presented in Fig. 2, where each point represents the average percentage of correct responses of the six subjects in successive blocks of 10 trials per subject (60 trials per point) relative to the three experimental sessions. The percentage of correct responses in the discrimination trials with vertical gratings of 1 c/deg (full points in Fig. 2) increased progressively during the first session from less than 70% in the first block of trials to more than 90% in the fourth to sixth block, and remained constant thereafter. The effects of trial repetition on discrimination performance with the same pair of gratings were almost completely retained in the second session, run on the next day (Fig. 2, middle left) and completely retained in the third session (Fig. 2, bottom left).

In contrast, the effects of training on discrimination performance with vertical gratings failed to transfer to horizontal gratings (Fig. 2, top right): the percentage of correct responses dropped to levels near chance when the gratings were presented horizontally, and a further 40–50 trials were required for performance to improve to a 90% level.

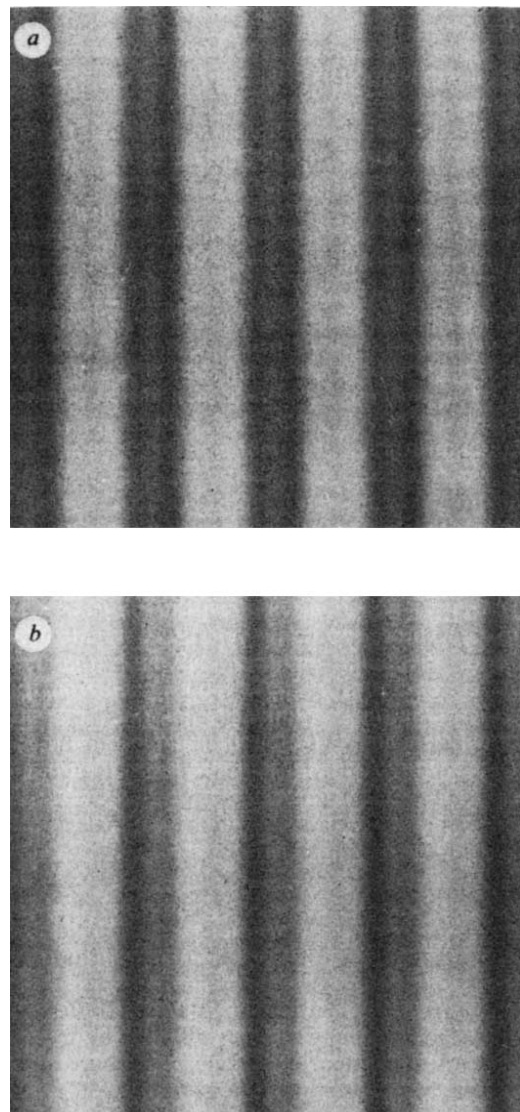


Fig. 1 Patterns to be discriminated: gratings *a* and *b*. The difference between the two patterns is obvious when they are viewed next to each other under steady illumination, but discrimination becomes more difficult when the patterns are presented briefly and in temporal sequence. The reader can appreciate this difficulty by illuminating the figure with a dim light flash.

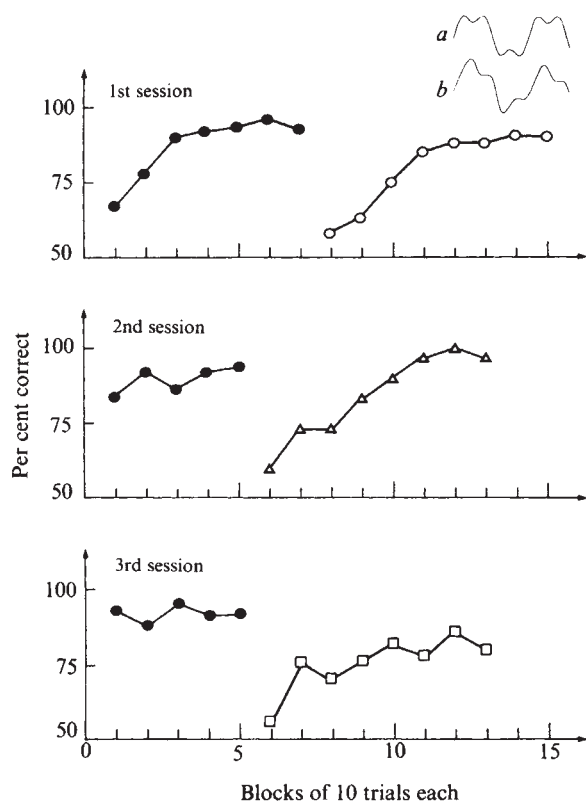


Fig. 2 Percentage of correct forced-choice discriminations of gratings *a* and *b* relative to subsequent blocks of 10 trials each. Data were pooled for the six subjects (60 trials per point). The three graphs refer to three experimental sessions run on separate days. Full points, vertical gratings, fundamental spatial frequency 1 c/deg; open points, horizontal gratings, 1 c/deg (top); open triangles, vertical gratings, 0.5 c/deg; open squares, vertical gratings, 2 c/deg. Insert, luminance profiles of gratings *a* and *b*. In the first session the proportion of correct responses with vertical gratings increased from 0.8 to 1.0 for some subjects and from 0.5 to 0.9 for others. The inter-subject variability was similar in the subsequent runs, whenever a new pair of gratings was used. The changes in the proportions of correct responses from the last block with the practice gratings to the first block with different gratings are all highly significant ($P < 0.001$; two-tailed correlated *t*-test with six pairs of scores).

The same results were obtained in the following two sessions when the viewing distance was changed, so that the fundamental spatial frequency of the gratings varied from 1 to 0.5 c/deg (Fig. 2, middle right) or from 1 to 2 c/deg (Fig. 2, bottom right). In the latter case, performance levelled off at about 80% correct, probably because the third harmonic of the gratings was only slightly above threshold at this viewing distance.

In spite of the relatively small number of trials per point, the regular trend of the curves of Fig. 2 supplies unequivocal evidence that: (1) the ability to discriminate two complex gratings of different luminance profiles rapidly improves with training; (2) the effects of training on the discrimination of a given pair of gratings are retained, at least partially, from one day to the next; (3) the effects of training fail to transfer to gratings orientated at 90° or viewed from a distance differing by a factor of 2 (fundamental spatial frequency differing by 1 octave).

Experiments to be reported in detail elsewhere indicate that smaller changes in grating orientation ($\pm 30^\circ$) or spatial frequency (± 0.5 octave) do not prevent the effects of previous practice from transferring to the new stimuli.

Most observers were able to describe the cues used for discrimination, once they had acquired practice in the task. According to their reports, the patterns were discriminated either on the basis of the apparent sharpness of the bar edges (the bars of grating *a* looking more similar to the black and white

stripes of a square wave grating) or because of the detection of narrow dark and bright lines in grating *b* (corresponding to the points of lowest and highest luminance in the grating profile (Fig. 2)). These cues, however, were apparently of little use when the orientation of the gratings or the viewing distance was changed. Furthermore, as measurements were made with a forced-choice procedure, the drop in performance cannot have resulted merely from a change in criterion. The improvement in performance with practice is therefore perceptual learning.

The neural mechanism for the learning process remains unknown. However, the present findings unequivocally show that this process must occur at a stage of neural processing at which different orientations (and/or different spatial frequencies) are handled separately.

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1. Gibson, E. J. *Psychol. Bull.* **50**, 401 (1953).
2. Gibson, E. J. *Principles of Perceptual Learning and Development* (Appleton-Century-Crofts, New York, 1969).
3. Ramachandran, V. S. & Braddick, O. *Perception* **2**, 371 (1973).
4. McKee, S. P. & Westheimer, G. *Percept. Psychoph.* **24**, 258 (1978).

T-cell specificity for H-2 and Ir gene phenotype correlates with the phenotype of thymic antigen-presenting cells

Dan L. Longo & Ronald H. Schwartz

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

Experiments with chimaeric animals have demonstrated that the H-2 restriction specificity and immune response (Ir) gene phenotype of the T cell is acquired during development in the thymus. The mechanism by which this process occurs is unclear. One level of obligate expression of H-2 and Ir gene products is on the surface of antigen-presenting cells (APCs) which come from bone marrow precursors. We have now examined the turnover of APCs in the thymuses of F_1 -parent (P) radiation-induced bone marrow chimaeras and found that APCs of donor phenotype appear at about 2 months after reconstitution. If the peripheral T-cell population is depleted after this time, new T cells emerging from the parental thymus (containing F_1 APCs) behaved like F_1 T cells, suggesting that cells from the bone marrow can influence thymic-directed T-cell differentiation. The thymic APC is an attractive candidate to play such a part in the development of the T-cell repertoire.

Experiments with (responder $\times$ nonresponder) ($R \times NR$) F_1 mice¹ and $NR \rightarrow (R \times NR) F_1$ chimaeric mice²⁻⁸ have demonstrated that, for at least one major category of Ir genes, the APC must be of responder genotype to initiate Ir-controlled responses. In contrast, the T cell need not possess the responder allele(s) in its genome as long as it has developed in a responder environment²⁻⁸. Grafting of nonresponder animals with responder thymus tissue has shown that the thymus is responsible for the phenotypic alteration⁹. Anti-Ia antiserum blocking studies have shown that the genotypic nonresponder T cell becomes a phenotypic responder by learning to recognize Ia molecules of the responder type plus antigen on the surface of the APC¹⁰. The mechanism by which the chimaeric T-cell population acquires the ability to recognize responder Ia as self in the thymus is unclear, but seems to be a fundamentally different process from tolerance induction or depletion of specific alloreactivity¹¹. It is presumed that the maturation of the

Table 1 Most thymic APCs in 1-year-old chimerae are of bone marrow donor origin

Source of APCs	T cells									
	B10.A			B10.A(18R)			(B10.A × B10.A[18R])F ₁			
	Non-pulsed	PPD-pulsed	Δ	Non-pulsed	PPD-pulsed	Δ	Non-pulsed	PPD-pulsed	ΔPPD	GLΦ-pulsed
B10.A thymus	2,753 ± 290	114,693 ± 7,429	111,940	102,315 ± 9,120	99,667 ± 5,881	-2,648	3,126 ± 289	77,364 ± 5,929	74,238	5,024 ± 611
B10.A × B10.A[18R]F ₁ thymus	73,286 ± 4,589	139,221 ± 6,722	55,935	58,246 ± 4,021	124,694 ± 10,755	66,448	3,371 ± 410	145,320 ± 9,798	141,949	107,446 ± 7,420
B10.A → (B10.A × B10.A[18R])F ₁ chimaeric thymus	7,608 ± 575	120,941 ± 9,360	113,333	89,437 ± 7,349	98,623 ± 10,071	9,186	2,916 ± 308	86,225 ± 6,824	82,309	10,372 ± 668

Lymph node T cells from antigen-primed mice were collected by passage over nylon wool columns. Thymus cells which had been spun in a discontinuous BSA gradient and given 2,000R *in vitro* were used as sources of APCs. The APCs were pulsed with 60 μg purified protein derivative of *Mycobacterium tuberculosis* (PPD) or 100 μg poly-(Glu⁵⁵-Lys³⁶-Phe⁹)_n (GLΦ) and 10⁵ cells were added to 4 × 10⁵ lymph node T cells in flat-bottomed microtitre plates. Antigen-specific proliferation was measured by assessing the ³H-thymidine incorporated into DNA on day 5 of culture by antigen-pulsed versus non-pulsed APCs. The data are expressed as c.p.m. ± s.e.m. for triplicate cultures or as Δ, the difference between antigen-pulsed and non-pulsed cultures. B10.A thymus cells present PPD to B10.A and F₁ T cells but not to B10.A(18R) T cells. F₁ thymus cells present PPD to all three types of T cells, and present GLΦ to the F₁ T cells. The APCs from the thymus of B10.A → (B10.A × B10.A[18R])F₁ chimerae present PPD well to B10.A and F₁ T cells but poorly to B10.A(18R) T cells. This feature, along with the minimal amount of GLΦ-specific proliferation they induce in F₁ T cells, suggests that most of the APCs in the chimaeric thymus are of B10.A donor origin.

Table 2 Donor type APCs appear in the thymuses of F₁ → P chimerae about 2 months after reconstitution

Source of APCs	T cells							
	B10.A lymph node			(B10.A × B10)F ₁ lymph node				
	Non-pulsed	DNP-OVA-pulsed	ΔDNP-OVA	Non-pulsed	DNP-OVA-pulsed	ΔDNP-OVA	GLΦ-pulsed	ΔGLΦ
B10.A spleen	1,188 ± 219	41,326 ± 4,008	40,138	2,089 ± 175	33,648 ± 3,161	31,559	2,204 ± 211	115
(B10.A × B10)F ₁ spleen	9,745 ± 781	30,998 ± 2,268	21,253	1,957 ± 236	60,523 ± 5,989	58,566	18,762 ± 2,009	16,805
1-week chimaeric thymus (Top BSA fraction)	1,698 ± 267	72,141 ± 1,519	70,443	1,716 ± 242	58,262 ± 6,102	56,546	1,889 ± 310	173
B10.A spleen	2,317 ± 197	62,819 ± 5,583	60,502	4,862 ± 1,010	40,146 ± 3,547	35,284	4,889 ± 526	27
(B10.A × B10)F ₁ spleen	12,862 ± 698	44,710 ± 3,972	31,848	5,029 ± 619	72,167 ± 6,739	67,137	29,433 ± 2,579	23,404
2-week chimaeric thymus	2,744 ± 255	121,085 ± 10,126	118,341	5,217 ± 830	108,679 ± 11,044	103,462	5,765 ± 624	548
B10.A spleen	1,814 ± 228	29,487 ± 2,269	27,673	6,914 ± 655	34,926 ± 2,968	28,012	7,475 ± 927	561
(B10.A × B10)F ₁ spleen	15,127 ± 1,641	28,889 ± 1,523	13,762	5,792 ± 529	49,611 ± 5,012	43,819	18,267 ± 1,398	12,475
1-month chimaeric thymus	3,168 ± 358	39,607 ± 3,242	36,439	6,345 ± 804	87,431 ± 8,667	81,086	5,831 ± 1,021	0
B10.A spleen	4,276 ± 398	29,657 ± 1,881	25,381	3,724 ± 629	26,717 ± 2,279	22,993	3,998 ± 447	274
(B10.A × B10)F ₁ spleen	21,684 ± 1,971	31,804 ± 3,026	10,120	4,024 ± 312	41,618 ± 3,926	37,594	15,066 ± 981	11,042
2-month chimaeric thymus	47,815 ± 4,226	89,762 ± 8,549	41,947	4,256 ± 491	63,295 ± 6,440	59,039	14,311 ± 575	10,055

B10.A animals were immunized with 10 μg DNP-ovalbumin (DNP-OVA) and (B10.A × B10)F₁ animals with the same dose of DNP-OVA and 30 μg GLΦ, all in complete Freund's adjuvant, in both hind footpads. Eight days later, lymph node T cells were obtained by passing lymph node cells over nylon wool columns. 4 × 10⁵ cells were plated with 2 × 10⁵ antigen-pulsed or non-pulsed spleen cells or thymus APCs and antigen-specific proliferation was measured by subtracting the ³H-thymidine c.p.m. on day 5 of culture of non-pulsed spleen or thymus cells from the counts obtained with antigen-pulsed spleen or thymus cells. Pulsing of cells was accomplished by preincubating *in vitro* irradiated (2,000R) spleen or thymus cells (10⁷) with 100 μg of GLΦ or 60 μg of DNP-OVA in 1 ml of RPMI 1640 + 10% fetal calf serum for 1 h at 37 °C and washing thoroughly to remove any soluble antigen. Thymic APCs were obtained by taking the cells at the top interface of a discontinuous BSA gradient consisting of 35, 29, 27 and 11% layers which had been spun at 25,000g for 30 min. The ability of thymic APCs from (B10.A × B10)F₁ → B10.A chimerae to present GLΦ to F₁ immune T cells was assessed at varying times after reconstitution. The data show that at about 2 months after reconstitution, cells from the thymus of such chimerae are able to present GLΦ to F₁ immune T cells and elicit an MLR in B10.A T cells, suggesting the presence of donor phenotype F₁ APCs.

T cell to self-recognition in the thymus involves the T cell 'seeing' Ia products on the surface of some cell in the thymus. There are two cell types which have been proposed to have this role, thymic epithelial cells^{11,12} and thymic macrophages¹³. Some studies have shown Ia molecules on the surface of thymic epithelial cells; however, the cell source of these molecules has not been conclusively demonstrated¹⁴. Macrophages have been found in the thymus and such cells have been reported to affect *in vitro* T-cell maturation¹⁵. Recently, we have demonstrated the existence of APCs in the thymus by a functional assay. The ability of such thymic APCs to present antigen can be eliminated by treatment with anti-Ia antiserum plus complement (data not shown). Thus, thymic APCs bear Ia antigens on their surface. We felt that a parsimonious theory of H-2 restriction and Ir gene function might be proposed if APCs in the thymus could be shown to be involved in T-cell maturation. Studies on the APC in spleen⁴, peritoneal cavity⁴ and skin (Langerhans cells)^{15,16} have shown that its precursors originate in bone marrow. Thus, the theories on the cell responsible for exposing the T cell to what is self can be distinguished because, in a radiation-induced bone marrow chimera, the radioresistant thymic epithelium should be replaced in turnover processes by host genotype epithelial precursors, while old or dying APCs should be

replaced by APCs of donor marrow origin, if thymic APCs are similar to APCs in other sites.

To see if thymic APCs are replaced by bone marrow precursors, we examined the phenotype of the thymic APCs in parent (P) low responder → F₁ responder chimerae. Such chimerae were made by reconstituting irradiated poly-(Glu⁵⁵-Lys³⁶-Phe⁹)_n (GLΦ) responder (B10.A × B10.A[18R])F₁ mice with T-cell-depleted GLΦ low responder B10.A bone marrow. Thymic APCs were isolated from B10.A → (B10.A × B10.A[18R])F₁ chimerae 1 yr after they were created by fractionating the thymus cells on a discontinuous bovine serum albumin (BSA) gradient (see Table 2 legend)¹³. As shown in Table 1, APCs from such chimeraic thymuses presented GLΦ poorly to F₁ immune T cells but successfully initiated a secondary proliferative response to purified protein derivative of *Mycobacterium tuberculosis* (PPD) in both F₁ and B10.A T cells but not in B10.A(18R) T cells. Also, note the presence of a large mixed lymphocyte reaction (MLR) when B10.A(18R) T cells were used as responders, as well as the negligible MLR made by B10.A T cells in response to the thymic APCs of the B10.A → (B10.A × B10.A[18R])F₁ chimerae. These data support the notion that donor phenotype B10.A APCs predominate in the thymus, thus suggesting that thymic APCs in

Table 3 T-cell restriction specificity in $F_1 \rightarrow P$ chimaeras is converted from host to donor phenotype by ATS and cortisone treatment

	Medium	DNP-OVA	GL Φ	(T, G)-A--L	Cytochrome c
(1) A $\times$ B $\rightarrow$ A	984 $\pm$ 106	31,612 $\pm$ 2,886	1,123 $\pm$ 146	1,076 $\pm$ 221	9,751 $\pm$ 868
(2) A $\times$ B $\rightarrow$ A 3 months after ATS+cortisone	10,820 $\pm$ 3,647	56,724 $\pm$ 4,228	26,420 $\pm$ 7,156	34,073 $\pm$ 686	17,221 $\pm$ 535
(3) A $\times$ B $\rightarrow$ B	4,781 $\pm$ 1,023	61,811 $\pm$ 6,673	5,400 $\pm$ 721	51,808 $\pm$ 4,782	4,722 $\pm$ 562
(4) A $\times$ B $\rightarrow$ B 3 months after ATS+cortisone	2,198 $\pm$ 642	28,971 $\pm$ 1,777	20,208 $\pm$ 2,431	—	—
(5) B6A $F_1 \rightarrow$ B10.A	5,129 $\pm$ 289	81,368 $\pm$ 7,249	5,490 $\pm$ 671	5,017 $\pm$ 1,011	15,894 $\pm$ 964
(6) B6A $F_1 \rightarrow$ B10.A 3 months after ATS+cortisone	7,055 $\pm$ 837	90,208 $\pm$ 8,266	54,816 $\pm$ 4,173	41,725 $\pm$ 3,268	20,188 $\pm$ 1,863
(7) B10.A 3 months after ATS+cortisone	6,711 $\pm$ 278	61,827 $\pm$ 3,159	5,682 $\pm$ 495	6,653 $\pm$ 491	16,922 $\pm$ 957
(8) B6A F_1 3 months after ATS+cortisone	4,249 $\pm$ 368	81,889 $\pm$ 10,218	69,814 $\pm$ 5,117	57,431 $\pm$ 3,272	14,827 $\pm$ 966

Eight months after irradiating parental B10.A (A) and B10 (B) mice with 900–950 R and reconstituting them with T-cell-depleted (B10.A $\times$ B10) F_1 (A $\times$ B) or B6A F_1 bone marrow, the $F_1 \rightarrow P$ chimaeras were immunized with 10 μ g DNP-OVA, 30 μ g GL Φ , 50 μ g (T,G)-A--L lot MC6 and 100 μ g pigeon cytochrome c (gift of Drs E. Margoliash and M. Ultee) in complete Freund's adjuvant in both hind footpads. Eight days later, draining popliteal and inguinal lymph node cells were passed over nylon wool columns and 4×10^5 cells were plated in flat-bottomed microtitre plates with soluble antigen (20 μ g ml $^{-1}$ DNP-OVA, 100 μ g ml $^{-1}$ GL Φ , (T,G)-A--L and pigeon cytochrome c). Responses were quantified by incorporation of 3 H-thymidine into DNA on day 5 of culture. Lines 1, 3 and 5 show responses of (A $\times$ B) $F_1 \rightarrow$ B10.A, (A $\times$ B) $F_1 \rightarrow$ B10 and B6A $F_1 \rightarrow$ B10.A chimaeras, respectively. F_1 T cells are capable of making responses to all of the antigens tested, but when they develop in a parental environment they are restricted to responses characteristic of that parent. (B10 is a responder to DNP-OVA and (T,G)-A--L and a nonresponder to GL Φ and cytochrome c; B10.A is a responder to DNP-OVA and cytochrome c and a nonresponder to GL Φ and (T,G)-A--L). However, when cage partners of host restricted $F_1 \rightarrow P$ chimaeras were treated with 0.6 ml of a 1:10 dilution of ATS and 5 mg of cortisone acetate, both intraperitoneally, 3 months later their lymph node T cells behave like F_1 cells, that is they are responders to GL Φ , (T,G)-A--L and cytochrome c (lines 2, 4 and 6). Lines 7 and 8 show that ATS and cortisone treatment does not alter the phenotype of normal parental or F_1 T cells.

radiation-induced bone marrow chimaeras are ultimately replaced by bone marrow precursors.

The turnover of APCs in the skin and spleen after radiation is markedly different. Splenic APCs disappear within 3–4 days after irradiation and are totally replaced by bone marrow precursors about 7 days after irradiation⁴. Skin APCs, on the other hand, take 65–80 days to be replaced by bone marrow precursors^{15,16}. In an effort to determine when thymic APCs in the radiation-induced bone marrow chimaera begin to be replaced by cells of donor origin, we made (B10.A $\times$ B10) $F_1 \rightarrow$ B10.A chimaeras and assayed the antigen presentation function of their thymic APCs at varying times after reconstitution. As shown in Table 2, between 1 week and 1 month after reconstitution, $F_1 \rightarrow P$ thymic APCs were unable to present GL Φ to F_1 immune T cells and no significant MLR was elicited from parental type B10.A T cells. The ability of thymic APCs to present GL Φ to F_1 immune T cells first appeared 2 months after reconstitution, at the same time that a substantial MLR in B10.A T cells was seen. These findings suggested that, at 2 months after reconstitution, donor F_1 APCs were detectable in the thymus of $F_1 \rightarrow P$ chimaeras.

If thymic APCs are important in the development of the T-cell restriction repertoire, then the T cells emerging from a chimaeric thymus after its APCs have become donor genotype should have the donor's restriction specificity. However, T cells, even in 1-year-old chimaeras, behave as though they are restricted to host and not donor phenotype. One possible explanation for this is that the initial T cells maturing in the thymus of a radiation chimaera dominate the peripheral T-cell pool for most of the remainder of the animal's life. If so, new T cells maturing after turnover of APCs in the thymus would make a negligible contribution to the T-cell pool and thus no conversion of the responsiveness of chimaeric T cells would be seen. To test this, we took $F_1 \rightarrow P$ chimaeras 8 months after reconstitution at a time when their T cells were demonstrated to be restricted to cooperative interactions with cells bearing host (parental) major histocompatibility complex (MHC) products, treated them with anti-thymocyte serum (ATS) and cortisone to deplete them of peripheral and thymic T cells, and allowed their parental thymus containing F_1 APCs (Table 2) to repopulate the periphery with T cells. Three months after ATS and cortisone treatment, and 11 months after reconstitution, the mice were immunized and their lymph node T cells assayed. As shown in Table 3, (B10.A $\times$ B10) $F_1 \rightarrow$ B10.A, (B10.A $\times$ B10) $F_1 \rightarrow$ B10, and B6A $F_1 \rightarrow$ B10.A chimaeric T cells lost their restrictions to host genotype interactions and made substantial proliferative responses to GL Φ , (T, G)-A--L and pigeon cytochrome c (lines 1–6). They behaved like F_1 T cells. In control experiments,

treatment of normal B10.A or B6A F_1 mice with ATS and cortisone had no effect on the Ir gene phenotype of their T cells 3 months later (lines 7, 8).

The most straightforward interpretation of these data is that self-H-2 is learned in the thymus by T cells upon interaction with MHC products on thymic APCs. The first T cells generated in an $F_1 \rightarrow P$ chimaera encounter parental APCs during maturation and become restricted to parental H-2 specificities. Those T cells emerging from a parental thymus, which has become repopulated with donor F_1 APCs, become restricted to F_1 H-2 specificities. This correlation between the phenotype of the thymic APC and the T-cell restriction repertoire suggests that thymic APCs may be involved in the development of T-cell self-specificity. Alternatively, it is possible that the results demonstrate that in $F_1 \rightarrow P_A$ chimaeras, a suppression mechanism exists¹⁷ which prohibits detection of P_B and F_1 -reactive T cells and which is sensitive to ATS and cortisone treatment. Once this suppression mechanism is blocked, P_B and F_1 -reactive T cells can be expanded and detected. Our own preliminary experiments have failed to document any suppression of F_1 T-cell function in $F_1 \rightarrow P$ chimaeras. Therefore, we favour the former hypothesis.

The process by which the genome and the developmental milieu interact to produce the exquisitely precise T-cell recognition structure(s) which possesses dual specificities for antigen and MHC products remains an enigma. Evidence has been presented here that suggests that MHC products expressed on the APC in the thymus encountered by the T-cell precursor at a critical stage in its development may be responsible for its subsequent restricted cell interactions.

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- Yano, A., Schwartz, R. H. & Paul, W. E. *Eur. J. Immun.* **8**, 334–338 (1978).
- Kappler, J. W. & Marrack, P. *J. exp. Med.* **148**, 1510–1522 (1978).
- Hodes, R. J., Hathcock, K. S. & Singer, A. *J. Immun.* **123**, 2823–2829 (1979).
- Longo, D. L. & Schwartz, R. H. *J. exp. Med.* **151**, 1452–1467 (1980).
- Zinkernagel, R. M. *et al. J. exp. Med.* **148**, 592–610 (1978).
- Von Boehmer, H., Haas, W. & Jerne, N. K. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2439–2442 (1978).
- Matsunaga, J. & Simpson, E. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6207–6212 (1978).
- Billings, P., Burakoff, S. J., Dorf, M. E. & Benacerraf, B. *J. exp. Med.* **148**, 352–359 (1978).
- Hedrick, S. M. & Watson, J. *J. exp. Med.* **150**, 646–659 (1979).
- Longo, D. L. & Schwartz, R. H. *Fedn Proc.* **39**, 1127 (1980).
- Zinkernagel, R. M. *Immun. Rev.* **42**, 224–270 (1978).
- Jenkinson, E. J., Owen, J. J. T. & Aspinall, R. *Nature* **284**, 177–179 (1980).
- Beller, D. I. & Unanue, E. *J. Immun.* **121**, 1861–1866 (1978).
- Rouse, R. V., van Ewijk, W., Jones, P. P. & Weissman, I. L. *J. Immun.* **122**, 2508–2515 (1979).
- Frelinger, J. G., Hood, L., Hill, S. & Frelinger, J. A. *Nature* **282**, 321–324 (1979).
- Katz, S. I., Tamaki, K. & Sachs, D. H. *Nature* **282**, 324–326 (1979).
- Smith, F. I. & Miller, J. F. A. P. *J. exp. Med.* **151**, 246–251 (1980).

Cloned lines of natural killer cells

Gunther Dennert

Department of Cancer Biology, The Salk Institute for Biological Studies, PO Box 85800, San Diego, California 92138

A class of cytotoxic effector cells has recently been discovered^{1,2} in normal and immunodeficient nude mice that lyse a wide range of targets of both tumour and non-tumour origin. Unlike other lymphocytes, these natural killer (NK) cells are present at high levels without prior priming and have no immunological memory. Because NK cells act without prior sensitization, they could have a crucial role in immunosurveillance, providing a first defence against arising neoplastic cells. The question of NK-cell specificity is not well understood; results so far suggest that they may recognize specific antigens on their targets. Thus unlabelled competitor cells can inhibit the cytotoxic reaction of NK cells³. Furthermore, target-effector binding can be inhibited by isolated target structures⁴. Experiments in which the lysis of mouse and human NK-sensitive targets was competitively inhibited with target suggested that the targets recognized by NK cells are not common ones. To study this important aspect, permanent lines and clones of NK cells have been established *in vitro*. I report here a successful attempt to do this, and show that sublines display the same specificity spectrum as NK cells from normal cells even after recloning. This proves that antigen receptors on NK cells are not clonally distributed but leaves open the two possibilities that NK cells either recognize common structures on NK-sensitive targets or carry many receptors each with a different specificity. Cell-surface markers of NK lines distinguished them from allospecific thymus-derived lymphocyte cell lines through their lack of Lyt-1 and Lyt-2 antigen.

Athymic mice are known to be deficient in functional T cells, yet do not experience a particularly high incidence of tumours. This observation has been attributed to the high NK activity of lymphoid cells from nude mice. These properties made spleen cells from nude mice an appropriate starting material to establish NK cell lines. A number of different supernatants conditioned by spleen cells stimulated with mitogens were tested as tissue culture media. Results showed that media derived from normal spleen cells stimulated with $10 \mu\text{g ml}^{-1}$ concanavalin A (Con A)⁵ were superior to all others tested (data not shown) and supported the survival of effector cells from BALB/c *nu/nu* spleens able to lyse YAC-1 targets (Table 1, expt 1 and ref. 6). Fourteen individual spleen cell cultures were set up in conditioned medium, 12 of which were from BALB/c *nu/nu* mice and 2 from normal BALB/c mice. The cultures were fed weekly by replacing the medium and checked for cell growth. Twelve of the cultures grew progressively and were assayed after 4 months for their cytotoxic activity on YAC-1. Most cultures showed high cytolytic activity in a 6-h assay at attacker-to-target ratios (*a/t*) between 1:1 and 30:1 (Table 1, expt 2).

In subsequent experiments, all cultures were assayed on a panel of target cells, comprising YAC-1 and its relatively NK-resistant derivative, YAC-8 (provided by Drs M. Hansson and R. Kiessling), B10 ME and its relatively NK-resistant parent, BCN (provided by Drs P. Patek, J. Collins and M. Cohn), the C58 lymphoma RIG1 and various spleen blast cells induced by bacterial lipopolysaccharide (LPS). There was no detectable difference between the various cultures, culture 11 being a representative example (Table 1, expt 3). There was no lysis on the H-2^k thymic lymphoma RIG1 or in LPS spleen blast cells, which are excellent targets for cytotoxic T cells⁷. Strong lysis on YAC-1 and significantly lower lysis on YAC-8 were seen. Similarly to the YAC-1, YAC-8 pair, there was higher lysis on B10 ME than on BCN. Further experiments showed that lysis is

independent of Con A because the assays could be performed in the presence of α -methyl mannoside (data not shown). These results showed that these cultures display NK-like activity. Cloning of two of the lines was attempted after they had been in culture for about 4 months. Earlier cloning was not feasible since the cultures seemed to go through a crisis at about 2–3 months after culture initiation. I chose culture 11, derived from a BALB/c mouse and culture 12 derived from a BALB/c *nu/nu* mouse, because they showed somewhat better growth and viability than the others. Both cultures were conditioned to growth at low cell density by seeding 1,000 cells in 0.2 ml into microtitre wells.

Once the cells had grown up they were transferred into bottles and cloning was attempted at 100, 30, 10 and 3 cells per well. Unfortunately, line 12 grew poorly and did not clone below 100 cells per well. In contrast, line 11 showed much better cloning efficiency in that about 30% of the wells with 10 cells per well grew. Individual colonies were grown up and assayed for cytotoxicity on YAC-1 targets. From Table 1, expt 4, it is seen that the sublines have various degrees of cytolytic activity. For instance, subline 11.2 has only 10% cytotoxicity at an *a/t* ratio of 3:1 while sublines 11.5 and 11.13 have 78% and 75% cytotoxicity respectively. Four individual sublines were assayed on four different targets at different *a/t* ratios and compared

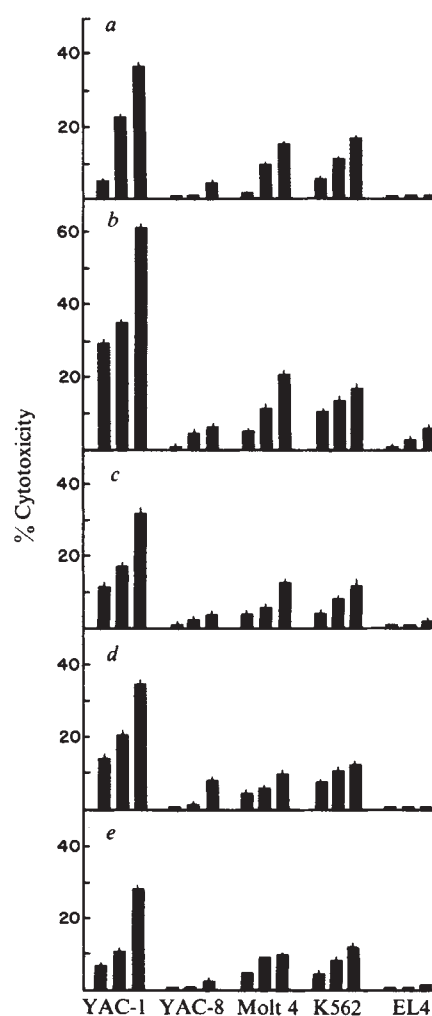


Fig. 1 BALB/c spleen cells (a) and the NK sublines 11.1 (b), 11.3 (c), 11.5 (d) and 11.11 (e) were assayed for their cytolytic activity on YAC-1 (H-2^a), YAC-8 (H-2^a), Molt 4, K562 and EL4 (H-2^b) targets. Molt 4 and K562 were grown in RPMI 1640 10% FCS and EL4 was grown in D-minimal essential medium plus 10% horse serum. *a/t* ratios were 25:1, 50:1, 100:1 for the spleen cells and 1:1, 2:1, 5:1 for the clones (from left to right). The 8 h time point is given.

Table 1 Cytolytic activity of various NK lines and sublines

	Effector cells	Target	% Cytotoxicity at <i>a/t</i> ratios							
			1:1	3:1	4:1	6:1	10:1	20:1	30:1	100:1
Expt 1 (3 h)	BALB/c	YAC-1					21 ± 0.3		36 ± 1.1	54 ± 2.1
	<i>nu/nu</i>	YAC-1					13 ± 0.4		22 ± 0.2	36 ± 0.9
	<i>nu/nu</i> cultured	YAC-1					20 ± 0.9		40 ± 1.1	62 ± 0.8
Expt 2 (6 h)	Culture 1 <i>nu/nu</i>	YAC-1							<1	
	Culture 2 <i>nu/nu</i>	YAC-1							70 ± 0.5	
	Culture 2 BALB/c	YAC-1					64 ± 2.9			
	Culture 4 <i>nu/nu</i>	YAC-1					72 ± 3.1			
	Culture 7 <i>nu/nu</i>	YAC-1		14 ± 0.2						
	Culture 8 <i>nu/nu</i>	YAC-1					<1			
	Culture 9 <i>nu/nu</i>	YAC-1					23 ± 1.1			
	Culture 10 <i>nu/nu</i>	YAC-1							5 ± 0.2	
	Culture 11 BALB/c	YAC-1					43 ± 1.2			
	Culture 12 <i>nu/nu</i>	YAC-1		18 ± 1.1						
	Culture 13 <i>nu/nu</i>	YAC-1		52 ± 2.9						
	Culture 14 <i>nu/nu</i>	YAC-1	11 ± 1.1							
Expt 3 (6 h)	Culture 11	YAC-1			86 ± 0.6					
	Culture 11	YAC-8			44 ± 0.5					
	Culture 11	B10ME			32 ± 0.3					
	Culture 11	BCN			19 ± 2.9					
	Culture 11	R1·G1			1 ± 0.5					
	Culture 11	C57BL/6			<1					
	Culture 11	SJL			<1					
Expt 4 (5 h)	Cloned subline 11·1	YAC-1					66 ± 1.3			
	Cloned subline 11·2	YAC-1		10 ± 0.3						
	Cloned subline 11·3	YAC-1				57 ± 2.9				
	Cloned subline 11·4	YAC-1					60 ± 3.1			
	Cloned subline 11·5	YAC-1		78 ± 1.9						
	Cloned subline 11·6	YAC-1					41 ± 2.5			
	Cloned subline 11·7	YAC-1					61 ± 2.5			
	Cloned subline 11·9	YAC-1						63 ± 2.2		
	Cloned subline 11·10	YAC-1				46 ± 1.0				
	Cloned subline 11·11	YAC-1					63 ± 0.1			
	Cloned subline 11·13	YAC-1		75 ± 0.2						

Effector cells were assayed without prior culture or, spleen cells were cultured for 5 d in RPMI 1640 containing 5% fetal bovine serum (FCS), 5×10^{-5} M β -mercaptoethanol, 40% conditioned medium prepared from Con A stimulated spleen cells as described in ref. 5 for expt 1. For expt 2, cultures were fed with conditioned medium as above on a weekly basis for 4 months, then assayed on targets at one *a/t* ratio. For expt 3; after 4 months, culture 11 was assayed on YAC-1 (H-2^a), YAC-8 (H-2^b) B10ME (H-2^d), BCN (H-2^d), R1·G1 (H-2^b) and C57BL/6 and SJL blast cells induced by culture in bacterial lipopolysaccharide¹¹. All target cell lines were grown in RPMI 1640 10% FCS except R1·G1 which was grown in Dulbecco's minimal essential medium plus 10% horse serum. For expt 4, culture 11 was cloned by limiting dilution in Falcon (no. 3040) microtitre wells in conditioned medium as in expt 1 at 1,000 cells per well, grown up and recloned at 10 cells per well. Individual colonies were grown up in Falcon tissue culture tubes (2051), then transferred to Falcon bottles (3013) and fed on a weekly basis. Cytotoxicity was assayed as described in ref. 11 at various *a/t* ratios as indicated. The time of incubation is given for each experiment. % Cytotoxicity = [(experimental ⁵¹Cr release - spontaneous ⁵¹Cr release)/(maximal ⁵¹Cr release - spontaneous ⁵¹Cr release)] × 100.

with the cytolytic activity of normal BALB/c spleen cells (Fig. 1). It is seen that BALB/c spleen cells lyse YAC-1 cells very well but YAC-8 and EL4 are not or are very poorly lysed. Both Molt 4 and K562 showed intermediate lysis. Sublines 11·1, 11·5 and 11·11 all showed the same picture comparable to that of BALB/c spleen cells: good lysis of YAC-1, poor lysis on YAC-8 and EL4, and intermediate lysis of Molt 4 and K562. Other sublines which were similarly tested showed the same lytic specificity.

This result could be interpreted in three ways: first, our sublines, if they were true clones, might recognize a common structure on mouse and human NK targets. This conclusion would contradict the previous results obtained with competitive inhibition assays mentioned above^{3,4}. Second, NK effectors display more than one receptor each with different specificity on their cell surface. Consequently cloned effectors will lyse different targets using their respective specific receptors. Third, our sublines may not be true clones and consequently there may be different NK effectors with specific receptors in these sublines. Although cloning experiments cannot distinguish between the first two possibilities, the third possibility can be excluded by recloning. Sublines 11·3 and 11·8 were recently recloned at 1 cell per well. Assays on YAC-1, Molt 4, K562 and EL4 of five out of five clones from both sublines showed the same specificity as in Fig. 1 (data not shown). It is therefore likely that we are dealing with true clones. Hence, our NK sublines either recognize a common structure on these targets or display multiple receptors with different specificities. This finding does not exclude the possibility that there is another type of NK effector which expresses specific receptors clonally. In fact, Herberman *et al.*^{8,9} recently presented evidence that the NK

effectors may be heterogeneous in that both Thy 1⁺ and Thy 1⁻ NK effectors exist. It was therefore interesting to assay our lines and sublines with regard to their cell-surface markers. The original line 11 as well as three sublines were assayed for Thy 1, Lyt 1, Lyt 2 and T200 using monoclonal antibodies and flow microfluorimetry. Table 2 shows that line 11 as well as the three

Table 2 Cell surface markers of NK cell lines

Effector cells	Thy 1	T200	Lyt 1	Lyt 2
Culture 11*	+	+	-	-
Subline 11·3*	+	+	-	-
Subline 11·10*	+	+	-	-
Subline 11·11*	+	+	-	-
C·C3·11·75†	+	+	+	-
BALB/c anti-C3H T killer cells‡	+	+	-	+

The following rat spleen cell-mouse myeloma hybrids producing monoclonal antibodies were used: T24/31.7 specific for Thy 1 (isolated by Dr I. S. Trowbridge); I3/2.3 specific for T200 (ref. 10); 53·7·313 specific for Lyt-1 and 53·6·72 specific for Lyt-2 both (provided by Dr L. Herzenberg).

*NK lines were assayed for cell-surface markers by flow microfluorimetry (FMF) using a rabbit anti-rat immunoglobulin as staining reagent.

†C·C3·11·75 is an allospecific H-2^d anti-H-2^b T cell line established in November 1975 from an MLR culture and maintained since then by allogeneic stimulation¹¹. Assay by FMF as for the NK cell lines.

‡BALB/c anti-C3H T killer cells were raised in a primary MLR and the effects of monoclonal antibodies assayed by complement dependent lysis.

sublines are Thy 1⁺, T200⁺ but Lyt 1⁻ 2⁻. Since Ly 5 is a polymorphism of T200 glycoprotein¹⁰, by inference these NK cells are Ly 5⁺. The lack of Lyt 1 and Lyt 2 sets these cells clearly apart from allospecific lines of thymus-derived lymphocytes, which can be generated by a mixed lymphocyte reaction (MLR, ref. 11) and propagated by antigenic stimulation. Thus C·C3·11·75, a BALB/c (H-2^d) anti C3H (H-2^k) allospecific line¹¹, is clearly Lyt 1⁺. In control experiments the same anti-Ly reagents were shown to be specific; thus anti-Lyt-1 in contrast to anti-Lyt-2 does not lyse allospecific T-killer cells. The expression of large amounts of Thy-1 antigen on these NK-cell lines, as well as the presence of these NK cells in thymus-deficient nude

mice⁶, may suggest that these cells do not require differentiation in the thymus for their function. Furthermore, since Thy-1 negative NK cells have been described⁹, we may have to search for another class of NK cells, which may have different properties. Our cloned lines of NK cells may be instrumental in analysing the relationship of NK killing and antibody-dependent cell-mediated cytotoxicity. They also provide us with a powerful tool to unravel the nature of these cells and their significance in the immune surveillance against neoplasia.

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1. Roder, J. C. & Kiessling, R. *Scand. J. Immun.* **8**, 135 (1978).
2. Herberman, R. B. & Holden, H. T. *Adv. Cancer Res.* **27**, 305 (1978).
3. Kiessling, R., Klein, E. & Wigzell, H. *Eur. J. Immun.* **5**, 112 (1975).
4. Roder, J. C., Rosén, A., Fenyö, E. M. & Troy, F. A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1405 (1979).

5. Rosenberg, S. A., Spiess, P. J. & Schwarz, S. J. *Immun.* **121**, 1946 (1978).
6. Dennert, G. & Hyman, R. *Eur. J. Immun.* (in the press).
7. Dennert, G. & Hyman, R. *Eur. J. Immun.* **7**, 251 (1979).
8. Herberman, R. B., Nunn, M. E. & Holden, H. T. *J. Immun.* **121**, 304 (1978).
9. Mattes, M. J., Sharrow, S. O., Herberman, R. B. & Holden, H. T. *J. Immun.* **123**, 2851 (1979).
10. Omary, B., Trowbridge, I. & Scheid, M. J. *exp. Med.* (in the press).
11. Dennert, G. *Nature* **277**, 476 (1979).

Involvement of the B-lymphoid system in chronic myelogenous leukaemia

Paul J. Martin*, Vesna Najfeld†, John A. Hansen*, Grace K. Penfold‡, Robert J. Jacobson§|| & Philip J. Fialkow†

* Division of Medical Oncology, Fred Hutchinson Cancer Research Center, Puget Sound Blood Center, Seattle, Washington 98104 and the Department of Medicine, University of Washington, Seattle, Washington 98195

† Medical Service, Veterans Administration Medical Center, Seattle, Washington 98108 and the Department of Medicine, University of Washington, Seattle, Washington 98195

‡ Department of Hematology, South African Institute for Medical Research and School of Pathology, University of the Witwatersrand, Johannesburg, South Africa

§ Department of Medicine, University of the Witwatersrand and Baragwanath Hospital, Johannesburg, South Africa

Studies with glucose-6-phosphate dehydrogenase (G6PD) isoenzymes have demonstrated that chronic myelogenous leukaemia (CML) is a clonal disorder of pluripotent haematopoietic stem cells which are capable of differentiation to myeloid cells, monocytes, erythrocytes and platelets¹. It has been observed recently in G6PD heterozygous patients with chronic phase CML that the non-E-rosetting lymphocytes were restricted to a single enzyme type, indicating that some lymphoid cells must also arise from the leukaemic clone². Surface or cytoplasmic immunoglobulin could be detected in up to 46% of the cells of these isolated non-T-lymphocyte populations, which suggested that cells from the CML clone were capable of differentiating into B lymphocytes. To investigate this further, we established Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines derived from patients with CML and studied chromosomes and G6PD to determine whether progenitor B lymphocytes for any of the cell lines had originated from the CML clone. We report here direct evidence that immunoglobulin-synthesizing B lymphocytes can arise from the CML stem cell clone.

Cells for this study were obtained from patient M.K., a woman with Philadelphia chromosome (Ph¹)-positive CML in the chronic phase and known to be heterozygous at the G6PD locus. Peripheral blood leukocytes (PBL) from M.K. were infected with EBV³ and then dispensed into multiple wells of a microtray (0.5–1.0 × 10⁶ cells per 0.2 ml). Cells from each transformed microculture well were expanded individually as separate cell lines. Preliminary results established that nearly all of the cell

lines derived in this fashion from M.K. displayed either A or B G6PD. Furthermore, with some exceptions, a single class of cytoplasmic immunoglobulin heavy chain and light chain were identified in each line. Thus, at the time of study, nearly all of the cell lines were monoclonal. With conventional methods, it is not possible to determine simultaneously the immunoglobulin class, G6PD type and chromosomal composition of a single B cell. With these B-cell lines, however, it was possible to examine the progeny of a single cell for all three markers, and thus infer the phenotype of the progenitor cell for each of the lines.

In nine of the cell lines derived from M.K., 93% or more of the cells were Ph¹-positive (Table 1). In the cells of each of these lines, there was a translocation involving chromosome 7, as well as numbers 22 and 9, the two chromosomes usually involved in the Ph¹ rearrangement, giving the karyotype 46, XX, t(7; 9; 22) (q11 or 21; q34.0; q11.1) (Fig. 1). The same karyotypic rearrangement was present in the patient's unstimulated peripheral blood metaphases for at least 3.5 yr.

Only type B G6PD was detected in each Ph¹-positive cell line, identical to what was found in the blood cell leukaemic clone of this G6PD heterozygous patient². In further studies, the differentiative capacity of the progenitors for four of the Ph¹-positive B-cell lines was examined by identifying the classes of immunoglobulin synthesized by each cell line. The data are presented in Table 1. In these lines, one-third or more of the cells were positive for cytoplasmic immunoglobulin. Moreover, the classes of immunoglobulin differed from line to line. Two cell lines were tested for the expression of Ia antigen and EBV nuclear antigen (EBNA), MK 12.4 and MK 24.6, and each line was positive for both markers.

For unexplained reasons it has not been possible to maintain these cell lines *in vitro* for long periods of time. All of the Ph¹-positive cell lines have lost viability after culture for 3 weeks to 5 months. Ph¹-negative B-lymphoblastoid lines derived from M.K. have been maintained in identical conditions without difficulty, and seem to have the same growth potential as B-lymphoblastoid cell lines derived from normal donors.

Ph¹-positive cell lines have been derived from at least two other CML patients^{4,5}. One of these, NALM-1, is a pre-B-lymphoid cell line derived from the peripheral blood of a patient with CML in blast crisis. The other one, K562, was established from the pleural effusion of a patient with CML in blast crisis. K562 is capable of *in vitro* differentiation to haemoglobin-producing erythroid cells⁶. Both of these cell lines are EBNA negative, unlike the MK 12.4 and MK 25.6 cell lines described here.

We have used EBV to derive from a patient with CML a series of B-lymphoblastoid cell lines which originated from the leukaemic clone. A similar approach has been reported recently by Hurley *et al.* for the study of chronic lymphocytic leukaemia

|| Present address: Division of Hematology, Department of Medicine, Georgetown University School of Medicine, Washington, DC 20007.

(CLL)⁷. Anti-immunoglobulin idiotype reagents were used to demonstrate that some B-lymphoblastoid cell lines obtained from CLL patients had originated from the CLL clone. In cytogenetic studies, the cell lines which expressed the CLL idiotype were aneuploid, whereas lymphoblastoid cell lines which did not express the CLL idiotype were cytogenetically normal. Thus, EBV infection can transform some progeny of the malignant clone both in patients with CLL and in those with CML.

The finding of Ph¹ in B-lymphoblastoid cell lines derived from our patient directly demonstrates that precursor cells originating from the CML clone are capable of differentiating into immunoglobulin-producing B lymphocytes. Evidence supporting this conclusion has also been provided by the six reported cases of CML in blast crisis in which the majority of blasts were of the pre-B phenotype (cytoplasmic immunoglobulin positive, surface immunoglobulin negative)⁸⁻¹⁰. Although the immunoglobulin synthesis by these cells could reflect the differentiation potential of the original CML clone, the possibility cannot be excluded that immunoglobulin synthesis by a highly malignant (that is, blast phase) CML clone resulted from alterations in the genome that occurred as a secondary consequence of malignancy. Whatever the mechanism that resulted in immunoglobulin synthesis, the cells of these patients represented a monoclonal expansion of malignant blasts, as indicated by studies of the immunoglobulin light chain⁸.

The cell lines described here provide evidence that cells of the malignant clone can differentiate into B lymphocytes even during the chronic phase of CML. These cell lines produce different classes of immunoglobulin, indicating further that this differentiation process is not limited to a single immunoglobulin species. This in turn suggests that immunoglobulin restriction is not fixed in stem cells of the CML clone, and that these cells may be responsive to the induction factors which normally regulate B-lymphoid differentiation. The diversity of differentiation seen for the CML clone during the chronic phase, demonstrated here by unrestricted immunoglobulin synthesis in Ph¹-positive stem cells, contrasts with the uniform immunoglobulin expressed by cells during blast crisis. Therefore, immunoglobulin restriction presumably occurs at some stage in differentiation between the pluripotent stem cell involved in the chronic phase of CML and the more differentiated blast cells present in the acute phase of the disease.

Table 1 Ph¹, cytoplasmic immunoglobulin and G6PD analyses of Ph¹-positive cell lines

Line	No. of cells studied	Ph ¹ *		Cytoplasmic immunoglobulin (% of cells positive)					G6PD
		No. of Ph ¹ -positive cells		μ	α	γ	κ	λ	
MK 3.10	70/70			31	0	0	0	33	B
MK 12.4	80/80			20	0	0	57	0	B
MK 12.7	95/95			0	17	0	0	75	B
MK 25.6	110/110			87	0	0	0	82	B

For chromosome studies, Ph¹ was enumerated in conventionally stained metaphases. After screening 30–50 mitoses, an additional 20–60 G-banded metaphases were analysed. More than 93% of cells from each of the B-lymphoblastoid cell lines were 46,XX,t(7;9;22)(q11 or 21;q34.0;q11.1) (see Fig. 1). Cytoplasmic immunoglobulin was stained as described with rhodamine-conjugated F(ab')₂ fragments specific for μ , γ , α , κ and λ (ref. 14). These reagents were provided by Dr S. M. Fu. Cells were examined with a Leitz Ortholux Two microscope with a 200 W mercury lamp and Ploemopak two filter system. A minimum of 300 cells were counted. For G6PD determination, cell lysates made by freezing and thawing were subjected to starch gel electrophoresis and the relative activity of the isoenzyme bands was estimated visually. The sensitivity of this technique enables a minor enzyme activity component of 5% to be detected¹⁵.

* The data for the five other Ph¹-positive lines in which immunoglobulin was not studied are 60/60 for line MK-229.1, 78/80 for 12.15, 79/80 for 12.19, 28/30 for 31.2 and 30/30 for 12.23.

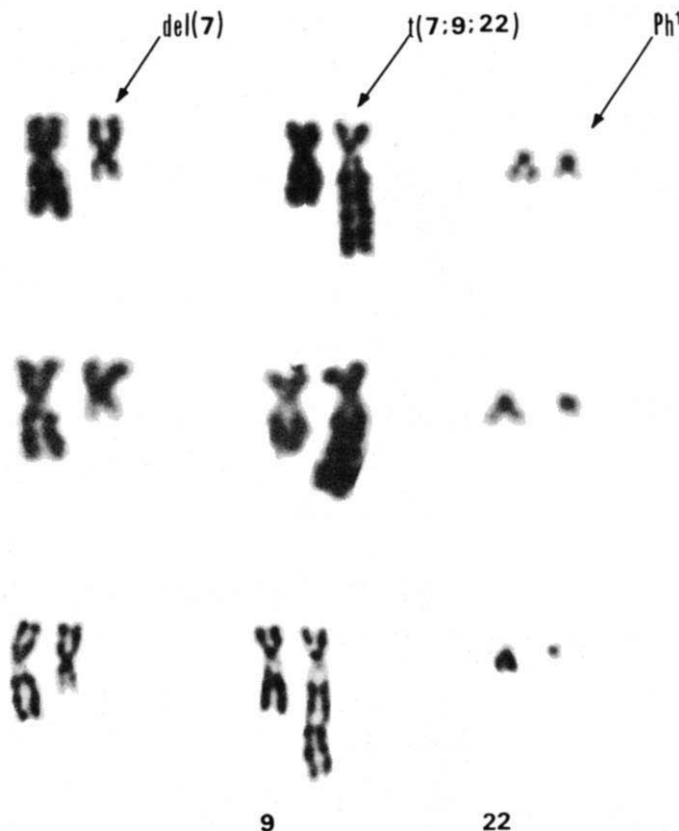


Fig. 1 Partial banded karyotypes of Philadelphia chromosome-positive B-lymphoblastoid cell lines. Colchicine ($1 \mu\text{g ml}^{-1}$) was added to the cell lines for the final three hours of culture, prior to fixation and staining. Slides were then subjected to a modified 'ASG' banding technique to obtain the banded karyotype of the cells¹¹. Paris Conference¹² and its supplement¹³ were used for chromosome nomenclature.

The Ph¹-positive B-lymphoblastoid cell lines derived from patient M.K., taken together with previous studies of this and similar patients^{1,2}, demonstrate that there exists in man a haematopoietic stem cell capable of B-lymphoid, as well as granulocytic, erythrocytic and megakaryocytic differentiation, and that this pluripotent stem cell is involved in CML.

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1. Fialkow, P. J., Jacobson, R. J. & Papayannopoulou, T. *Am. J. Med.* **63**, 125–130 (1977).
2. Fialkow, P. J., Denman, A. M., Jacobson, R. J. & Lowenthal, M. N. *J. clin. Invest.* **62**, 815–823 (1978).
3. Miller, G. & Lipman, M. *Proc. natn. Acad. Sci. U.S.A.* **70**, 190–194 (1973).
4. Lozzio, C. B. & Lozzio, B. B. *Blood* **45**, 321–334 (1975).
5. Minowada, J., Koshida, H., Janossy, G., Greaves, M. F. & Bollum, F. J. *Leukaemia Res.* **3**, 261–266 (1979).
6. Anderson, L. D., Jokinen, M. & Gahrberg, C. G. *Nature* **278**, 364–365 (1979).
7. Hurler, J. N., Fu, S. M., Kunkel, H. G., Chaganti, R. S. K. & German, J. *Nature* **283**, 76–78 (1980).
8. Leiben, T. W., Hozier, J., Minowada, J. & Kersey, J. H. *New Engl. J. Med.* **301**, 144–147 (1979).
9. Vogler, L. B., Crist, W. M., Vinson, A. S., Brattain, M. G. & Coleman, M. S. *Blood* **54**, 1164–1170 (1979).
10. Greaves, M. F. *et al. Leukaemia Res.* **3**, 181–191 (1979).
11. Sumner, A. T., Evans, H. J. & Buckland, R. A. *Nature new Biol.* **232**, 31–32 (1971).
12. Paris Conference: Standardization in Human Cytogenetics (The National Foundation—March of Dimes BD, OAS VIII, 1972).
13. Paris Conference (1971) & Suppl. (1975) (The National Foundation—March of Dimes BD, OAS XI No. 9, 1975).
14. Hijmans, W., Schuit, H. J. R. & Klein, F. *Clin. exp. Immun.* **4**, 457–472 (1969).
15. Fialkow, P. J. *Ann. hum. Genet.* **37**, 39–48 (1973).

Enzymatic assembly of slow reacting substance

B. A. Jakschik & L. H. Lee

Department of Pharmacology, Washington University Medical School, St Louis, Missouri 63110

When basophils or mast cells are stimulated by a specific antigen they release chemical mediators, including a potent bronchoconstrictor, slow reacting substance of anaphylaxis (SRS-A). The structure of SRS from a mouse mastocytoma^{1,2} and rat basophilic leukaemia (RBL-1) cells³ has been identified as a thioether of arachidonic acid and glutathione^{2,3} [not a thioether of cysteine as was originally thought¹]. SRS has been named leukotriene (LT) C and may be formed by a novel lipoxygenase pathway which also synthesizes 5,6-oxido-7,9,11,14-icosatetraenoic acid (LTA) and 5,12-dihydroxy-6,8,10,14-icosatetraenoic acid (LTB)^{1,4}. Homogenates of RBL-1 cells, when incubated with ¹⁴C-arachidonic acid, form 5-hydroxy-icosatetraenoic acid (5-HETE) and 5,12-dihydroxy- and 5,6-dihydroxy-icosatetraenoic acid⁵. The latter is the spontaneous breakdown product of the labile intermediate LTA⁴. Formation of both compounds is stimulated by calcium. We have now produced biologically active SRS in a cell-free system generated from RBL-1 cells. Glutathione was essential for SRS synthesis and calcium stimulated its formation.

The experiments were carried out with 10,000g supernatant of RBL-1 homogenates^{5,6}. The buffer used for the preparation contained 50 mM phosphate pH7, 1 mM EDTA, 0.1% gelatin and 14 μ M indomethacin. Briefly, RBL-1 cells were washed once with buffer, resuspended at 5×10^7 cells per ml and homogenized on a Tekmar Tissumizer. The homogenate was centrifuged at 10,000g for 20 min. The formation of hydroxyacids from ¹⁴C-arachidonate was determined by TLC as described previously^{5,6}. SRS synthesis was monitored on the isolated, superfused guinea pig ileum⁷. Figure 1 shows the results of a typical SRS experiment. Addition of glutathione proved to be essential for the formation of SRS activity. No such activity was

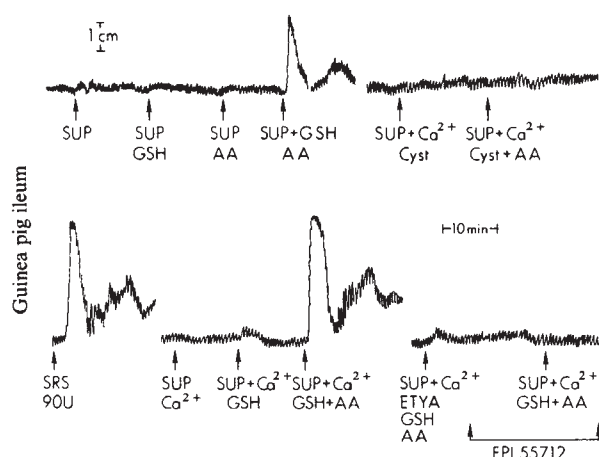


Fig. 1 SRS synthesis by a cell-free system. 10,000g supernatant of RBL-1 homogenates was incubated at 37 °C for 15 min with the agents indicated at the following concentrations: reduced glutathione (GSH) 1×10^{-3} M, calcium (Ca^{2+}) 2×10^{-3} M, cysteine (Cyst) 1×10^{-3} M, arachidonic acid (AA) 1.6×10^{-5} M, icosatetraenoic acid (ETYA) 6.7×10^{-5} M. 100 μ l of the incubation mixture was tested on the superfused guinea pig ileum⁷. FPL 55712 was applied to the tissue at a concentration of 2×10^{-7} M. The SRS standard was generated from RBL-1 cells as described previously⁹.

Table 1 Modulation of leukotrienes

	SRS Activity* (% max)	5,12-DHETE (% c.p.m.)†	5-HETE (% c.p.m.)†
Sup + Ca^{2+} + GSH + AA	100	6.1 ± 1.4	15.0 ± 7.0
Sup + GSH + AA	43.6 ± 8.7	—	—
Sup + Ca^{2+} + AA	0	25.9 ± 4.7	12.1 ± 1.5
Sup + AA	0	1.7 ± 0.5	2.3 ± 1.5
Boiled Sup + Ca^{2+} + GSH + AA	0	—	—
Boiled Sup + Ca^{2+} + AA	—	1.0 ± 0.4	1.2 ± 0.4
% Inhibition			
ETYA‡	96.5 ± 3.5	90.6 ± 3.5	75.2 ± 2.9
FPL 55712	95.7 ± 2.2	—	—

* The 10,000g supernatant (Sup) from RBL-1 cell homogenates was incubated and tested as in Fig. 1. The size of the contraction was evaluated by determining the area under the curve. The contraction obtained in the presence of Ca^{2+} 2×10^{-3} M, glutathione (GSH) 1×10^{-3} M and arachidonic acid (AA) 1.6×10^{-5} M, was arbitrarily taken as 100% ($n = 5-7$).

† The 10,000g supernatant from RBL-1 cell homogenates was incubated as described in Fig. 2 legend. Quantification was achieved by scraping and liquid scintillation counting ($n = 3$ or 4).

‡ Inhibition with icosatetraenoic acid (ETYA) or FPL 55712 was performed as indicated in Figs 2 and 1, respectively. The data are presented as mean $\pm$ s.e.m.

produced when cysteine was substituted for glutathione. The SRS activity was readily blocked by its specific receptor antagonist FPL 55712 (Fig. 1, Table 1). These data confirm earlier reports^{2,3} that glutathione and not cysteine is part of the SRS molecule. The incorporation of glutathione rather than cysteine as such into SRS also agrees with our earlier observation with ³⁵S-cysteine⁸. A higher percentage of label was incorporated into SRS when RBL-1 cells were grown for 16 h in the presence of this amino acid as compared to brief exposure (1 h). These experiments had already suggested that cysteine was first incorporated into another compound before utilization in SRS synthesis.

However, the addition of glutathione to the incubation mixture reduced the formation of 5,12-DiHETE (Fig. 2, Table 1). This observation suggests the preferential formation of SRS over 5,12-DiHETE. Therefore, the SRS-forming enzyme, probably a glutathione transferase, has a higher affinity for the proposed common intermediate, the 5,6-epoxide, than the enzyme synthesizing 5,12-DiHETE. The production of SRS in the presence of low concentrations of glutathione (Table 1) and lack of SRS formation when boiled supernatant was used, strongly indicate that this SRS formation is enzymatic. These data, therefore, confirm the postulate¹ that SRS is formed by the same enzymatic pathway as LTB with a common intermediate, LTA.

Similar to 5-HETE and 5,12-DiHETE (Fig. 2, Table 1), SRS synthesis was stimulated by calcium (Fig. 1, Table 1). Therefore, calcium is important for this whole pathway. It is probably necessary for the first step since very little 5-HETE was formed in the absence of calcium (Fig. 2, Table 1). The enhancement of SRS formation by calcium does not appear to be quite as marked as for 5,12-DiHETE and 5-HETE. This may further indicate the preferential formation of SRS over 5,12-DiHETE. As can be seen in Table 1, there was a small, though not significant, increase in counts in the 5-HETE TLC area when no calcium was added to the incubation mixture as compared to incubations with boiled enzyme. Therefore, some hydroperoxide and LTA may be formed in the absence of exogenous calcium. The LTA is then preferentially converted to SRS. This would explain the formation of appreciable amounts of SRS in the absence of added calcium. Icosatetraenoic acid effectively inhibited the synthesis of all the products of the pathway (Figs 1, 2, Table 1).

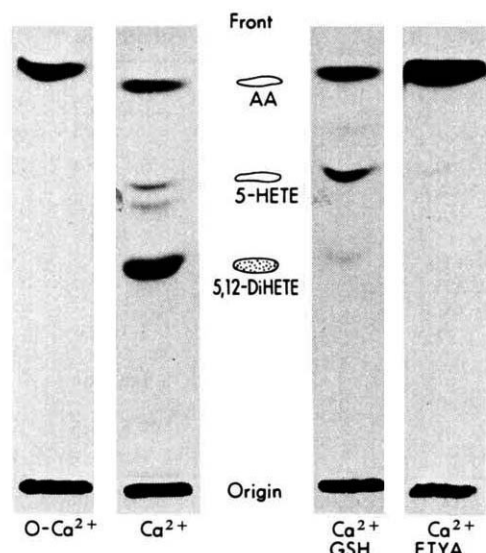


Fig. 2 Effect of glutathione and icosatetraynoic acid on 5,12-DiHETE formation. 10,000g supernatant (0.5 ml) was incubated with [^{14}C]arachidonic acid (AA) (200,000 c.p.m., specific activity 55 mCi mmol^{-1}) for 20 min at 37°C , with the agents indicated: calcium (Ca^{2+}) $2 \times 10^{-3} \text{ M}$, reduced glutathione (GSH) $1 \times 10^{-3} \text{ M}$, icosatetraynoic acid (ETYA) $6.7 \times 10^{-5} \text{ M}$. The reaction was stopped by precipitating the proteins with acetone. The pH was adjusted to 3.2–3.5 with 2 M formic acid. The mixture was extracted twice with 2 ml of chloroform. TLC was performed in solvent system A9, the organic phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:10; ref.¹⁰). Radioactive peaks were located by fluorography¹¹. Standards were visualized by iodine staining. The identity of 5,12-DiHETE has been established by gas chromatography–mass spectrometry⁵.

We are, therefore, now able to assemble SRS and the other LTs enzymatically in a cell-free system. The production of SRS by a cell-free system has not been reported previously despite many attempts. This preparation will enable us to study this pathway and its modulation in greater detail. The data presented here already indicate that in proper conditions SRS (LTC) is synthesized in preference to LTB. It will now be possible to investigate the requirements for each step in the pathway and determine what factors enhance or inhibit the formation of each product. The availability of the enzyme system in a cell-free preparation will allow the determination of substrate specificity for different fatty acids, glutathione analogues and sulphhydryl compounds. Therefore, use of this enzyme preparation provides a tool for identifying LT synthesis inhibitors. The discovery of a specific SRS (LTC) inhibitor would be of special significance since SRS is such a potent bronchoconstrictor.

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- Murphy, R. C., Hammarström, S. & Samuelsson, B. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4275–4279 (1979).
- Hammarström, S. *et al. Biochem. biophys. Res. Commun.* **91**, 1266–1272 (1979).
- Parker, C. W., Huber, M. M., Hoffman, M. K. & Falkenstein, S. F. *Prostaglandins* **18**, 673–686 (1979).
- Borgeat, P. & Samuelsson, B. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3213–3217 (1979).
- Jakschik, B. A., Sun, F. F., Lee, L. H. & Steinhoff, M. M. *Biochem. biophys. Res. Commun.* **95**, 103–110 (1980).
- Steinhoff, M. M. & Jakschik, B. A. *Biochim. biophys. Acta* **618**, 28–34 (1980).
- Jakschik, B. A., Lee, L. H., Shuffer, G. & Parker, C. W. *Prostaglandins* **16**, 733–748 (1978).
- Parker, C. W., Jakschik, B. A., Huber, M. G. & Falkenstein, S. F. *Biochem. biophys. Res. Commun.* **89**, 1186–1192 (1979).
- Jakschik, B. A., Kulczycki, A. Jr., MacDonald, H. H. & Parker, C. W. *J. Immun.* **119**, 618–622 (1977).
- Hamberg, M. & Samuelsson, B. *J. biol. Chem.* **241**, 257–263 (1966).
- Bonner, W. M. & Stedman, J. D. *Analyt. Biochem.* **89**, 247–256 (1978).

Behavioural effects and supersensitivity following nigral dopamine receptor stimulation

Michael R. Kozlowski, Steven Sawyer & John F. Marshall

Department of Psychobiology, University of California, Irvine, California 92717

Nigrostriatal dopamine (DA)-containing neurones have the potential to modulate neostriatal output at two levels. First, DA released from the terminals of the DA-containing pars compacta neurones can act on striatal DA receptors. In addition, these pars compacta cells can release DA from their dendrites within the nigra^{1–3}. Dendritically released DA could act at receptors located on these same cells ('autoreceptors')^{4,5} or at receptors which are located on other nigral elements, especially striatonigral afferent fibres^{4,6,7}. However, although the stimulation of striatal DA receptors produces clear behavioural effects, no behavioural consequence of nigral DA receptor stimulation has been shown^{8,9}. We report here that nigral DA receptor stimulation can produce clear behavioural effects in the appropriate circumstances. Unilateral intranigral injections of the DA receptor stimulant, apomorphine, produce clear contralateral turning in animals that (1) had been previously given intracerebral 6-hydroxydopamine (6-OHDA) injections, which extensively damage the ipsilateral nigral DA-containing cells, or (2) had been pretreated for 1 week with the DA receptor blocker, haloperidol. In addition, the turning produced by intranigral apomorphine in 6-OHDA-treated animals was much greater when the drug was given 14–17 days after the intracerebral neurotoxin than when it was given 4–7 days after, suggesting the development of supersensitivity to apomorphine in the nigra. These results suggest that the nigra must be considered as a site of action for drugs whose main effect is on the dopaminergic system.

Fifty male Sprague–Dawley rats (Simonsen Breeders) weighing 150–200 g had guide cannulae implanted 2 mm dorsal to the left substantia nigra. During the same surgery, 37 of these animals also received an injection of 6-OHDA (8 μg , Sigma) into the left ventral tegmental area (VTA) to destroy the mesotelencephalic DA-containing projection. Desmethylinipramine (15 mg per kg, intraperitoneally (i.p.)) was given 0.5 h before the 6-OHDA injection to protect brain noradrenaline-containing neurones. On days 4–17 postoperatively, all rats were given apomorphine injections through an inner cannula and were then placed in circular bowls where the number of complete rotations was counted for 1-min periods every 5 min for 1 h. After behavioural testing, the location of the cannula tracts was reconstructed from thionin-stained frontal sections of 20- μm thickness.

In agreement with the findings of others^{8,9}, injections of apomorphine (10 μg) into one substantia nigra of otherwise neurologically intact animals produced little behavioural effect. A small amount of contralateral turning was seen in two of the eight animals tested (Table 1). However, vigorous contralateral rotation occurred when intranigral apomorphine injections were given either 4–7 or 14–17 days after 6-OHDA injections into the ipsilateral VTA (Table 1). These 6-OHDA injections caused extensive ipsilateral DA depletion in both the forebrain (98, 80 and 82% decreases in the neostriatum, nucleus accumbens septi and olfactory tubercle, respectively) and the substantia nigra (90% decrease) when compared with the same structures in the opposite hemisphere. The turning produced by intranigral apomorphine in these DA-depleted animals was elicited in a dose-dependent manner (Fig. 1) and could be

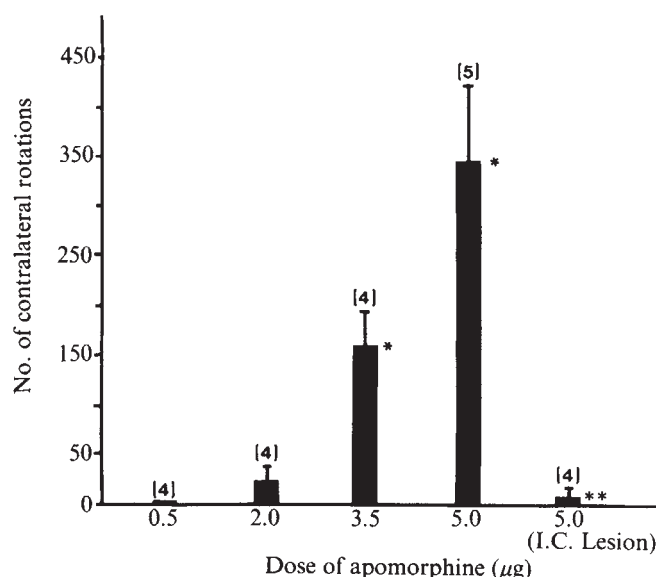


Fig. 1 Dose-response curve for contralateral rotation following intranigral apomorphine given 14–17 days after 6-OHDA treatment. The turning showed a significant dose dependence (F -test, $P < 0.001$). Numbers in parentheses indicate animals per group. I.C. = internal capsule. 6-OHDA was dissolved in 0.9% saline and 0.1% ascorbic acid ($2 \mu\text{g} \mu\text{l}^{-1}$) and injected at a rate of $1 \mu\text{l min}^{-1}$ at the following coordinates: A 2.6, L 1.0, V 7.8. All rats were given desmethylimipramine (15 mg per kg , i.p.) 0.5 h before 6-OHDA. Guide cannulae were implanted at A 2.3, L 2.0, V 5.8–6.3. Apomorphine was dissolved in 0.9% saline and 0.1% ascorbic acid and injected into the substantia nigra in a volume of $0.5 \mu\text{l}$ at a rate of $1 \mu\text{l min}^{-1}$ through an inner cannula which extended 2 mm ventral to the tip of the guide shaft. The rats were placed in circular bowls for 1 h following the apomorphine injection, where their rotational behaviour was recorded. Radio-frequency lesions (100 V , 30 s) were made in the internal capsule using a stainless steel dental broach insulated except for 0.6 mm at the tip, positioned at A 4.0, L 2.6, V 8.3. In addition to receiving intranigral apomorphine, the 6-OHDA-treated rats were tested for rotation in response to a low dose (0.25 mg per kg , i.p.) of apomorphine as an index of extensive unilateral DA depletion. Those rats that did not show vigorous contralateral rotation (≥ 200 turns) in the subsequent 1 h were not used in the experiment ($\sim 17\%$ of the 6-OHDA-treated rats). The DA content (in $\mu\text{g per g}$ wet tissue weight) of the striatum, nucleus accumbens septi, olfactory tubercle and substantia nigra in the right, non-injected hemisphere of 6-OHDA-treated rats were 6.7 ± 0.6 , 6.5 ± 1.7 , 4.3 ± 0.3 and 1.8 ± 0.5 , respectively.

* Significantly more turning than $0.5 \mu\text{g}$ group, Dunnett's t -test, $P < 0.05$;
** significantly less turning than $5.0 \mu\text{g}$ group, Student's t -test, $P < 0.001$.

blocked by a single systemic injection of the DA antagonist, haloperidol (2 mg per kg , i.p., Table 1). The amount of rotation produced by $5 \mu\text{g}$ of apomorphine was much greater 14–17 than 4–7 days after the 6-OHDA treatment (Table 1).

Rotation in response to intranigral apomorphine was also seen in animals chronically treated with haloperidol (5 mg per kg , i.p., daily for 1 week followed by a 3-day drug-free period, Table 1). Such chronic haloperidol treatment produces behavioural supersensitivity to systemically administered apomorphine^{10,11}. Interestingly, similar amounts of turning resulted from intranigral apomorphine injections even either after 7 days of haloperidol treatment or 4–7 days after an intracerebral 6-OHDA injection (Table 1).

To investigate the possibility that the turning produced by intranigral apomorphine was mediated by DA receptors located on striatonigral or pallidonigral axons, four animals were given small radiofrequency lesions along the course of these fibres in the medial tip of the internal capsule 11 days after an ipsilateral 6-OHDA injection into the VTA. These lesions nearly completely eliminated the rotation in response to intranigral apomorphine ($5 \mu\text{g}$, Fig. 1) 14 days after the 6-OHDA injection. They also reduced by 75% (t -test, $P < 0.01$) the turning elicited by the systemic injection of apomorphine (0.25 mg per kg , i.p.). Histological sections showed that the radiofrequency lesions were centred in the medial tip of the internal capsule at a level of A 3.9–A 4.1 (ref. 12). These lesions

extended an average distance of 1.0 mm in the antero-posterior plane, 0.6 mm in the medio-lateral plane and 1.2 mm in the dorso-ventral plane. In some brains, slight damage to the immediately adjacent zona incerta, ventral tegmentum and optic tract was found.

The effect of blocking receptors for γ -aminobutyric acid (GABA), a major transmitter in the striatonigral and pallidonigral pathways^{13,14}, with the GABA antagonist, picrotoxin (0.5 , 1.0 and 2.0 mg per kg , i.p.), was also examined. At 2.0 mg per kg , picrotoxin completely blocked the rotation induced by intranigral apomorphine ($5 \mu\text{g}$, Table 1), but the two lower doses had no effect on turning (data not shown).

Histological examinations confirmed that the cannula tips for apomorphine injection were located in the pars reticulata of the substantia nigra. In a separate group of six 6-OHDA-treated animals in which the cannula tips were placed in the pars compacta, intranigral apomorphine injections produced little or no turning.

The substantia nigra contains at least two types of DA receptor. One type, located on the DA-containing cells of the pars compacta, binds the DA antagonist ^3H -spiroperidol with high affinity but does not seem to be linked to an adenylate cyclase¹⁵. The second type, located predominantly on nigral afferents (striatonigral and possibly pallidonigral axons), is linked to adenylate cyclase but fails to bind ^3H -spiroperidol with high affinity^{13,15}. Three findings indicate that the latter type is important in producing turning after intranigral apomorphine. First, the extensive depletion of forebrain and nigral DA produced by the VTA 6-OHDA injection suggests a massive degeneration of the dopaminergic cells, so that the rotation elicited by intranigral apomorphine is unlikely to be mediated by these neurones. Second, lesions of an area of the internal capsule through which striatonigral fibres pass¹⁶ almost completely eliminate this rotation. Third, systemic picrotoxin blocks this rotation, possibly by blocking GABA receptors postsynaptic to the striatonigral fibres. However, because a high dose of picrotoxin was needed to block rotation (one-half the dose that is required to produce seizures), a non-physiological action of this drug cannot be ruled out.

Because DA can cause a release of GABA from nigral slices *in vitro*¹⁷, we suggest that the apomorphine-induced turning behaviour is likely to result from a local stimulation of GABA release from striatonigral axon terminals within the pars reticulata. This enhanced GABA release should then affect the

Table 1 Effects of 6-OHDA injections and pharmacological treatments on turning behaviour in response to intranigral apomorphine

Treatment	No. of animals	Apomorphine dose (μg)	No. of contralateral turns
1. Intranigral apomorphine only	8	10	1.4 ± 0.9
2. 6-OHDA injections 4–7 d before intranigral apomorphine	9	5	$48.2 \pm 14.0^{\dagger}$
3. 6-OHDA injection 14–17 d before intranigral apomorphine*	5	5	$344.6 \pm 76.6^{\ddagger}$
4. Chronic haloperidol (5 mg per kg , i.p. daily on days 5–11 postoperatively) followed by intranigral apomorphine on day 14	5	5	$33.6 \pm 10.2^{\dagger}$
5. 6-OHDA injection 4–7 d before and haloperidol (2 mg per kg , i.p.) 2 h before intranigral apomorphine	5	5	$6.8 \pm 2.9^{\ddagger}$
6. 6-OHDA injection 4–7 d before and picrotoxin (2 mg per kg , i.p.) 10 min before intranigral apomorphine	5	5	0 ± 0

* Results are from Fig. 1.

† Significantly different from group 1, t -test, $P < 0.05$.

‡ Significantly different from group 2, t -test, $P < 0.05$.

activity of nigral pars reticulata cells, which have been implicated in the production of rotational behaviour in other experiments^{18,19}.

In addition to these DA receptors located on pars compacta cells and on strionigral afferents, the nigra may contain additional DA binding sites, as combined lesions of nigrostriatal and strionigral projections do not totally eliminate nigral ³H-spiroperidol binding¹⁵. An action of apomorphine at these sites may contribute to the behavioural consequences of its intranigral injection, particularly if they are located on nigral output cells. However, the present report offers no evidence for an action of intranigral apomorphine at these sites.

The effects of nigral DA receptor stimulation are strikingly similar to those caused by the stimulation of DA receptors in the neostriatum, a terminal field for the ascending dopaminergic axons. First, although unilateral injections of apomorphine into either the nigra or the striatum produce little behavioural effect in neurologically intact animals (ref. 20 and unpublished findings), injections into either region do produce vigorous contralateral turning following ipsilateral damage of the ascending dopaminergic system, a single injection of reserpine or chronic haloperidol treatment (ref. 20 and unpublished findings). Second, the rotation resulting from both striatal and nigral apomorphine injections seems to be mediated by strionigral fibres (ref. 21 and the present findings). These similarities suggest the intriguing possibility that the axon terminals and dendrites of the dopaminergic pars compacta cells provide a parallel, dual control mechanism for altering the activity of strionigral fibres and, consequently, for controlling the nigral output cells of the pars reticulata.

It is particularly interesting that robust turning in response to intranigral apomorphine occurs in the 6-OHDA- or chronic haloperidol-treated animals, but that only occasional rotations are seen in otherwise untreated animals given the intranigral apomorphine injection. This suggests that the nigra shows enhanced sensitivity to apomorphine following these treatments. The sharply increased rotational response to intranigral apomorphine found between 4–7 and 14–17 days after the 6-OHDA injection parallels the increased behavioural sensitivity to systemically administered apomorphine which develops during this period^{22,23}.

One process thought to underlie the development of enhanced sensitivity to DA agonists in the neostriatum following damage to the dopaminergic system is a proliferation of postsynaptic DA receptors, as indicated by an increased number of ³H-haloperidol or ³H-spiroperidol binding sites^{24,25}. In the nigra, however, no increase in ³H-spiroperidol binding occurs after destruction of the dopaminergic system²⁴. This negative finding may reflect the fact that the high affinity ³H-spiroperidol binding sites are largely localized to the DA-containing cells¹⁵. A possible increase in nigral DA-sensitive adenylate cyclase activity after this lesion has not been investigated. Alternatively, the increased sensitivity to apomorphine may result from changes in the receptors postsynaptic to the strionigral axons. Indeed, chronic treatment with haloperidol increases ³H-GABA binding in the nigra^{26,27}.

These findings indicate that the nigra must be considered an important potential site of action of systemically administered drugs which act at DA synapses. Part of the contralateral rotation seen in unilateral 6-OHDA-treated rats given systemic apomorphine may be due to a direct nigral action of this drug. In addition, the nigra must be considered as a site of action of antipsychotic and anti-parkinsonian drugs. Finally, the development of an enhanced sensitivity to DA within the nigra may contribute to the behavioural recovery that occurs after subtotal damage to the ascending DA-containing fibres^{28,29}.

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1. Björklund, A. & Lindvall, O. *Brain Res.* **83**, 531–537 (1975).
2. Nicoullon, A., Cheramy, A. & Glowinski, J. *Nature* **266**, 375–377 (1977).
3. Hefti, F. & Lichtensteiger, W. *J. Neurochem.* **30**, 1217–1230 (1978).
4. Groves, P. M., Wilson, C. W., Young, S. J. & Rebec, G. V. *Science* **190**, 522–529 (1975).
5. Aghajanian, G. K. & Bunney, B. S. *Adv. Biochem. Pharmac.* **16**, 433–438 (1977).
6. Gale, K., Hong, J.-S. & Guidotti, A. *Brain Res.* **136**, 371–375 (1977).
7. McGeer, P. L., McGeer, E. G. & Innanen, V. T. *Brain Res.* **169**, 433–441 (1979).
8. Glick, S. D. & Crane, L. A. *Brain Res.* **156**, 380–381 (1978).
9. Kelley, P. Y. & Moore, K. E. *Neuropharmacology* **17**, 169–174 (1978).
10. Gianutsos, G., Drawbaugh, R. B., Haynes, M. D. & Lal, H. *Life Sci.* **14**, 887–898 (1974).
11. Von Voigtlander, P. F. *Fedn Proc.* **33**, 578 (1974).
12. König, J. F. R. & Klippel, R. A. *The Rat Brain: A Stereotoxic Atlas of the Forebrain and Lower Parts of the Brain Stem* (Krieger, New York, 1963).
13. Gale, K., Guidotti, A. & Costa, E. *Science* **195**, 503–505 (1977).
14. Jessell, T. M., Emson, P. C., Paxinos, G. & Cuello, A. C. *Brain Res.* **152**, 487–498 (1978).
15. Quick, M., Emson, P. C. & Joyce, E. *Brain Res.* **167**, 355–365 (1979).
16. Garcia-Munoz, M., Nicolaou, N. M., Tulloch, I. F., Wright, A. K. & Arbutnot, G. W. *Nature* **265**, 363–365 (1977).
17. Reubi, J.-C., Iversen, L. L. & Jessell, T. M. *Adv. Biochem. Psychopharmac.* **19**, 401–404 (1978).
18. Olanas, M. C., DeMontis, G. M., Mulas, G. & Tagliamonte, A. *Eur. J. Pharmac.* **49**, 233–241 (1978).
19. di Chiara, G., Porceddu, M. L., Morelli, M., Mulas, M. L. & Gessa, G. L. *Naunyn-Schmiedeberg's Archs Pharmac.* **306**, 153–159 (1979).
20. Ungerstedt, U., Butcher, L. L., Butcher, S. G., Andén, N.-E. & Fuxe, K. *Brain Res.* **14**, 461–471 (1969).
21. Marshall, J. F. & Ungerstedt, U. *Science* **198**, 62–64 (1977).
22. Ungerstedt, U. *Acta physiol. scand. Suppl.* **367**, 69–93 (1971).
23. Thornburg, J. E. & Moore, K. E. *J. Pharmac. exp. Ther.* **192**, 42–49 (1975).
24. Nagy, J. I., Lee, T., Seeman, P. & Fibiger, H. C. *Nature* **274**, 278–281 (1978).
25. Creese, I., Burt, D. R. & Snyder, S. H. *Science* **197**, 596–598 (1977).
26. Gale, K. *Neurosci. Abstr.* **5**, 71 (1979).
27. Gale, K. *Nature* **283**, 569–570 (1980).
28. Stricker, E. M. & Zigmond, M. J. *Progress in Physiological Psychology and Psychobiology* Vol. 6 (eds Sprague, J. M. & Epstein, A. M.) 121–180 (Academic, New York, 1976).
29. Marshall, J. F. *Brain Res.* **177**, 311–324 (1979).

The role of microvesicles in buffering $[Ca^{2+}]_i$ in the neurohypophysis

Jean J. Nordmann & Jean Chevallier*

INSERM U. 176, rue Camille Saint-Saëns, 33077 Bordeaux, France

* Institut de Biochimie Cellulaire & Neurochimie, 1 rue Camille Saint-Saëns, 33000, Bordeaux, France

It is well established that hormone release from neurosecretory nerve terminals is triggered by an increase of the cytoplasmic ionized calcium concentration which occurs after the arrival of action potentials¹. For the neurosecretory nerve terminals to recover their basal Ca^{2+} concentration, the calcium which has entered during depolarization must be extruded and/or accumulated in subcellular organelles or bound to some cytoplasmic proteins. Neurosecretory nerve endings are characterized by the presence of hormone-containing granules and microvesicles (earlier referred to as 'synaptoid' vesicles). The role of these microvesicles has been the subject of debate and has recently been reviewed². One of the most attractive hypotheses has been that these organelles represent the retrieved membrane of the granule after exocytosis. However, on stimulation of hormone release, the number of microvesicles does not increase, suggesting that they do not participate in the endocytotic process³. Using stereology as a quantifying method, we recently demonstrated a redistribution of microvesicles towards the release site during potassium-stimulated hormone release³. It is thus possible that microvesicles in neurosecretory nerve terminals might function as calcium accumulating organelles as suggested for the coated vesicles isolated from the cerebral cortex^{4,5}. We report here that a microvesicle-containing microsomal fraction isolated from neurosecretosomes (isolated neurosecretory nerve terminals) possesses an ATP-dependent calcium transporting system. This system enables the fraction to accumulate calcium ions when the external ionized calcium concentration is $<1 \mu M$, which is in the physiological range.

Bovine neural lobes (40–60 for each experiment) were collected from the local slaughterhouse and kept in ice-cold 0.3 M sucrose buffered with 10 mM HEPES, pH 7.2. The glands were carefully dissected out, minced with a pair of scissors and sliced on a MacIlwain tissue chopper at 0.5-mm intervals. The material was then homogenized at 800 r.p.m. in a Teflon homogenizer and the homogenate was centrifuged at 800 g_{av} for 10 min on a RC-5B Sorvall centrifuge using an HB-4 type rotor. The procedure was repeated with the resulting pellet (P_1) after a further homogenization. The two supernatants were mixed and centrifuged at 1,700 g for 20 min. The resulting pellet (P_2), which consisted mostly of neurosecretosomes⁶, was homogenized in 0.3 M buffered sucrose and the homogenate centrifuged at 26,000 g_{av} for 20 min. The supernatant from this was centrifuged at 100,000 g_{av} for 1 h. The resulting pellet (P_4) was practically devoid of mitochondria and neurosecretory granules, as judged by careful analysis of ultra-thin sections. P_4 was resuspended in 0.15 M KCl, 10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.2, and its protein content determined using the method of Lowry *et al.*⁷. In one experiment P_4 was fixed by the method described by Shaw and Morris⁸. The pellet consisted primarily of microvesicles and many of them contained precipitate indicating the presence of calcium. Figure 1 shows the time course of calcium uptake by the microvesicle-containing fraction at two different calcium concentrations. This uptake is ATP-dependent and is abolished by addition of the ionophore X537A. The amount of calcium taken up by the microvesicles-containing fraction is higher than that found for a microsomal fraction isolated from pinched-off presynaptic nerve terminals from rat brain cortex⁹. Neither the time course nor the amount of calcium uptake was affected by addition of mitochondrial inhibitors such as ruthenium red (10 μM), antimycin (0.05 $\mu g\ ml^{-1}$) or carbonyl cyanide trifluoromethoxyphenyl hydrazone FCCP (0.1 $\mu g\ ml^{-1}$). These results suggest that fragments of mitochondria are not responsible for the ATP-dependent calcium uptake of the microvesicle-containing fraction.

The effect on the Ca^{2+} uptake of the ionophore X537A (Fig. 1) is not due to the influence of an increased membrane

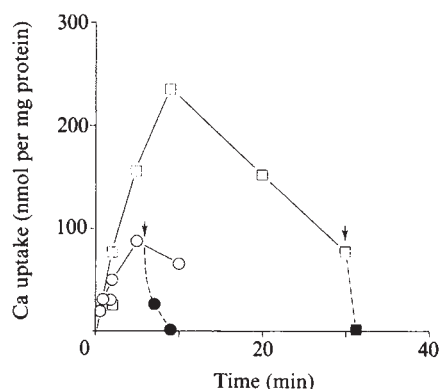


Fig. 1 ATP-dependent Ca^{2+} uptake in microvesicle-containing fraction isolated from bovine neurohypophysis. The preparation (200–300 μg protein ml^{-1}) was preincubated at 37 °C in 5 ml of a medium containing 150 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.2, ^{45}Ca -EGTA buffer. After 5 min, 4 mM ATP (final concentration) was added. At different times, 500- μl aliquots were withdrawn and immediately filtered through Millipore filters (0.22 μm pore diameter) as described in ref. 15. 100 μl of the resulting filtrates were counted in a liquid scintillation spectrophotometer. The calcium transport studies were always performed immediately after the isolation of the microvesicle-containing fraction because calcium uptake by the preparation was much lower 16–18 h after the final centrifugation. In some cases, a calcium binding of 10–20 nmol per mg of microsomal proteins was observed in the presence of 4 mM ADP. The ionized calcium concentrations were 0.66 μM (○) and 0.91 μM (□). At the time indicated by the arrow the ionophore X537A (10 μM final concentration) was added (●, ■).

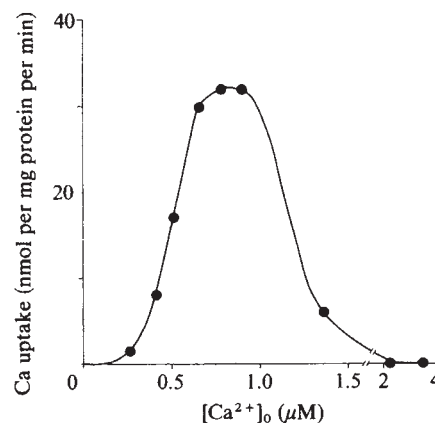


Fig. 2 Effect of free Ca^{2+} concentration on calcium uptake by the microvesicle-containing fraction. Experimental conditions as described in Fig. 1 legend. The calcium in the incubation medium was buffered to the indicated ionized Ca^{2+} concentration with EGTA (1 mM) by taking a Ca-EGTA stability constant of 1.86×10^{-7} M. The rate of calcium uptake was obtained from the initial slope of the kinetics illustrated in Fig. 1. The apparent K_m of the Ca^{2+} transport system lies between 0.4 and 0.6 μM . Curve drawn by eye.

permeability to Na^+ ions because (1) the microvesicles were incubated in a sodium-free medium, and (2) addition of sodium (20–40 mM) did not release calcium which had previously been accumulated. The lack of effect of sodium on the calcium accumulated by the microvesicle fraction shows that the preparation used in the present study is unlikely to be contaminated by plasma membranes because a Na^+ - Ca^{2+} exchange has been demonstrated in neurohypophyses isolated *in vitro*. Furthermore, the efflux of calcium from preloaded glands does not increase on addition of veratridine, an alkaloid known to increase the sodium conductance in nervous tissue. It thus seems that in neurosecretory nerve endings the release of calcium from the microvesicles is not modulated by the cytoplasmic sodium concentration.

The non-mitochondrial ATP-dependent Ca^{2+} transport system described above has a high affinity for calcium. After the calcium has been accumulated into the microvesicle-containing fraction, it can be released passively (Fig. 1). The rate of calcium release is a function of the amount of accumulated calcium. This release of calcium on overloading of the fraction has been observed in other systems¹⁰. In a series of experiments, aliquots of the microvesicle fraction were incubated in media containing different concentrations of ionized calcium. The rate and the amount of calcium taken up were measured as a function of $[Ca^{2+}]$ in the medium ($[Ca^{2+}]_0$) and the results are illustrated in Fig. 2. The apparent K_m for this transport system is 0.4–0.6 μM . These values lie within the apparent range of cytosolic Ca^{2+} ions, and are similar to those reported for a microsomal fraction isolated from lysed synaptosomes⁹. A pronounced inhibition of the Ca^{2+} transport activity is observed on increasing the free calcium concentration above 1 μM (Fig. 2). This phenomenon can be related to the competition between Ca^{2+} and Mg^{2+} for ATP utilization described in other systems¹¹.

The present study therefore provides a demonstration that, as suggested earlier^{3,12}, the microvesicles found in neurohypophysial nerve endings have the capacity to take up calcium and therefore may be involved in controlling the ionized cytoplasmic calcium concentration. The presence of calcium in some microvesicles has been demonstrated previously using the pyroantimonate technique¹². More recently, Shaw and Morris have been able to demonstrate, using a combined oxalate-pyroantimonate method, the presence of calcium in microvesicles in fixed neural lobes⁸. Microsomal calcium uptake by neurosecretory tissue has previously been shown but the data do not provide information about the origin (nerve cells or

pituicytes) of these microsomes¹³. The high affinity (Fig. 2) of the calcium transport system isolated from neurosecretosomes suggests that microvesicles may have an important role in intraterminal Ca^{2+} buffering. Although mitochondria take up calcium, their calcium transport system has a much higher K_m^{Ca} (ref. 14) than the microvesicle fraction. As the arrival of a burst of action potentials is likely to increase the intraterminal ionized calcium concentration from 10^{-7} to 5×10^{-6} M at the most, the microvesicles would buffer the $[\text{Ca}^{2+}]$ in this range. How the calcium accumulated in microvesicles is released from the nerve terminal is not known. Electron micrographs often show fusion of microvesicles with the plasmalemma and this suggests that the calcium could be released by exocytosis. Alternatively, calcium could be slowly released into the cytoplasm and long-term calcium homeostasis be controlled by some transport system located in the plasma membrane.

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1. Douglas, W. W. in *Handbook of Physiology* Sect. IV, 191–224 (Am. Physiol. Soc., Washington, DC, 1974).
2. Morris, J. F., Nordmann, J. J. & Dyball, R. E. J. *Int. Rev. exp. Path.* **18**, 1–95 (1977).
3. Morris, J. F. & Nordmann, J. J. *Neuroscience* **5**, 639–649 (1980).
4. Blitz, A. L., Fine, R. E. & Toselli, P. A. *J. Cell Biol.* **75**, 135–145 (1977).
5. Rahamimoff, H. & Spanier, R. *FEBS Lett.* **104**, 111–114 (1979).
6. Gratzl, M. *et al. Biochim. biophys. Acta* **470**, 45–57 (1977).
7. Lowry, O. H. *et al. J. Biol. Chem.* **193**, 265–275 (1951).
8. Shaw, F. D. & Morris, J. F. *Nature* **287**, 56–58 (1980).
9. Blaustein, M. P. *et al. J. gen. Physiol.* **72**, 15–41 (1978).
10. Chevallier, J. *et al. Biol. Cell* **30**, 103–110 (1977).
11. Hasselbach, W. *Prog. Biophys. molec. Biol.* **14**, 167–222 (1964).
12. Stoessel, M. E. *et al. Cell. Tissue Res.* **157**, 307–315 (1975).
13. Russell, J. T. & Thorn, N. A. *Acta physiol. scand.* **93**, 364–377 (1975).
14. Blaustein, M. P. *et al. Ann. N.Y. Acad. Sci.* **307**, 195–212 (1978).
15. Martonosi, A. & Feresos, R. *J. Biol. Chem.* **239**, 648–658 (1964).

Calcium localization in the rat neurohypophysis

F. D. Shaw* & J. F. Morris†

* Department of Anatomy, University of London King's College, London WC2R 2LS, UK

† Department of Human Anatomy, University of Oxford, Oxford OX1 3QX, UK

Neurosecretory nerve endings in the rat neurohypophysis release their hormones by exocytosis¹ subsequent to an influx of calcium from the external medium². The nerve endings are characterized by the presence of neurosecretory granules, mitochondria, occasional vacuoles, and a population of microvesicles similar in appearance to spherical synaptic vesicles. The function of the microvesicles has, for a long time, been uncertain. In view of evidence that coated microvesicles isolated from cerebral cortex are capable of ATP-dependent calcium accumulation³, a method has now been developed for the visualization of calcium in the neurohypophysis at the ultrastructural level. With this technique, calcium precipitates are consistently seen in the microvesicles, mitochondria and glial cell (pituicyte) nuclei. In addition, the pituicyte cytoplasm and perivascular space show a diffuse precipitate which can be removed by washing the tissue prior to fixation. The function of the microvesicles might therefore be to sequester calcium within the nerve endings.

The subcellular distribution of calcium in the rat neurohypophysis was studied by a modification of a technique previously described⁴. Adult male rats of the Porton–Wistar strain were anaesthetized with urethane and perfused with a glutaraldehyde fixative containing potassium oxalate, preceded in

some cases by a wash with potassium phosphate buffer containing dextran. The posterior pituitaries were dissected out, washed in an oxalate solution, and treated with an osmium tetroxide fixative containing potassium antimonate. The tissue was then washed in distilled water, dehydrated, and embedded for electron microscopy. Control tissue was fixed in solutions lacking oxalate and antimonate. Immersion in osmium/(pyro)antimonate fixatives has been widely used to visualize calcium in nervous and glandular tissue, but it gives relatively poor preservation of fine structure especially in the central nervous system^{5–7}. Initial fixation by perfusion with a rapidly penetrating aldehyde, as described here, allows much better preservation^{6,7}, at the same time immobilizing tissue calcium by precipitation with oxalate.

The neurohypophysis is largely composed of axons and terminals of magnocellular hypothalamic neurones, and of pituicytes. The unmyelinated neurosecretory axons are characterized by numerous dilations containing neurosecretory granules and mitochondria; neurosecretory nerve endings in addition contain abundant microvesicles, and contact a basement membrane adjacent to a fenestrated capillary⁸ (Fig. 1a). With the technique used, calcium is indicated by the electron-dense precipitate of antimony (Fig. 1b–d). When the neurohypophysis was perfused directly with the oxalate/glutaraldehyde fixative, a striking ultrastructural feature was the presence of abundant precipitates in the cytoplasm and nuclei of the pituicytes, to the extent that their processes were clearly outlined at low power (Fig. 1b). A dense precipitate was also present in the perivascular space, and more diffuse deposits were seen over the neurosecretory axons, swellings and terminals. The mitochondria of both neurones and glia were heavily stained; vacuoles usually contained precipitate and virtually every microvesicle contained a single, dense particle of precipitate.

This appearance was significantly altered if the tissue was washed with phosphate buffer solution containing dextran (which facilitates perfusion⁹) for 3–5 min before infusion of fixative. The perivascular space was thereby rendered completely free of precipitate and the diffuse precipitate over neurosecretory elements was also removed. However, precipitate remained localized within the microvesicles, whether they were dispersed (Fig. 1c) or in clusters (Fig. 1d) and in the vacuoles. Smooth endoplasmic reticulum elements also often contained the stain, and were occasionally seen in intimate association with the microvesicles (Fig. 1d). The mitochondria of both neurones and pituicytes (Fig. 1c, d) retained their precipitates, but the cytoplasmic precipitates in the pituicytes were removed and the precipitate in their nuclei considerably reduced in extent (Fig. 1d). Washing with buffer–dextran for 1–2 min produced an intermediate appearance, in which the perivascular space and pituicytes contained a precipitate which was less dense than when fixative was introduced immediately.

Various control experiments suggest that the precipitate visualized was due to calcium in the tissue. Inclusion of 5 mM EGTA in the oxalate wash before osmium/antimonate fixation resulted in the complete absence of deposits when the tissue was subsequently examined, and also demonstrated that they were not an artefact of the fixative procedure itself. Precipitate could also be removed from ultra-thin sections on grids by incubation in 5 mM EGTA at 60 °C for 1 h. This indicates that the deposits do not contain significant amounts of sodium or magnesium (which antimonate will precipitate at higher concentrations¹⁰).

The diffuse cytoplasmic precipitates seen in tissue exposed to glutaraldehyde/oxalate directly could conceivably be due to *in vivo* ambient levels of free calcium, though it would be difficult to explain their presence in greater amounts in the pituicytes. It seems more likely that they result from the influx of calcium ions from the extracellular space as the tissue dies¹¹. However, glial cells can apparently store calcium¹², and pituicytes are capable of taking up radioactive calcium when stimulated with potassium¹³. The presence of gap junctions between pituicytes has led to the suggestion that they might modulate neurosecretory

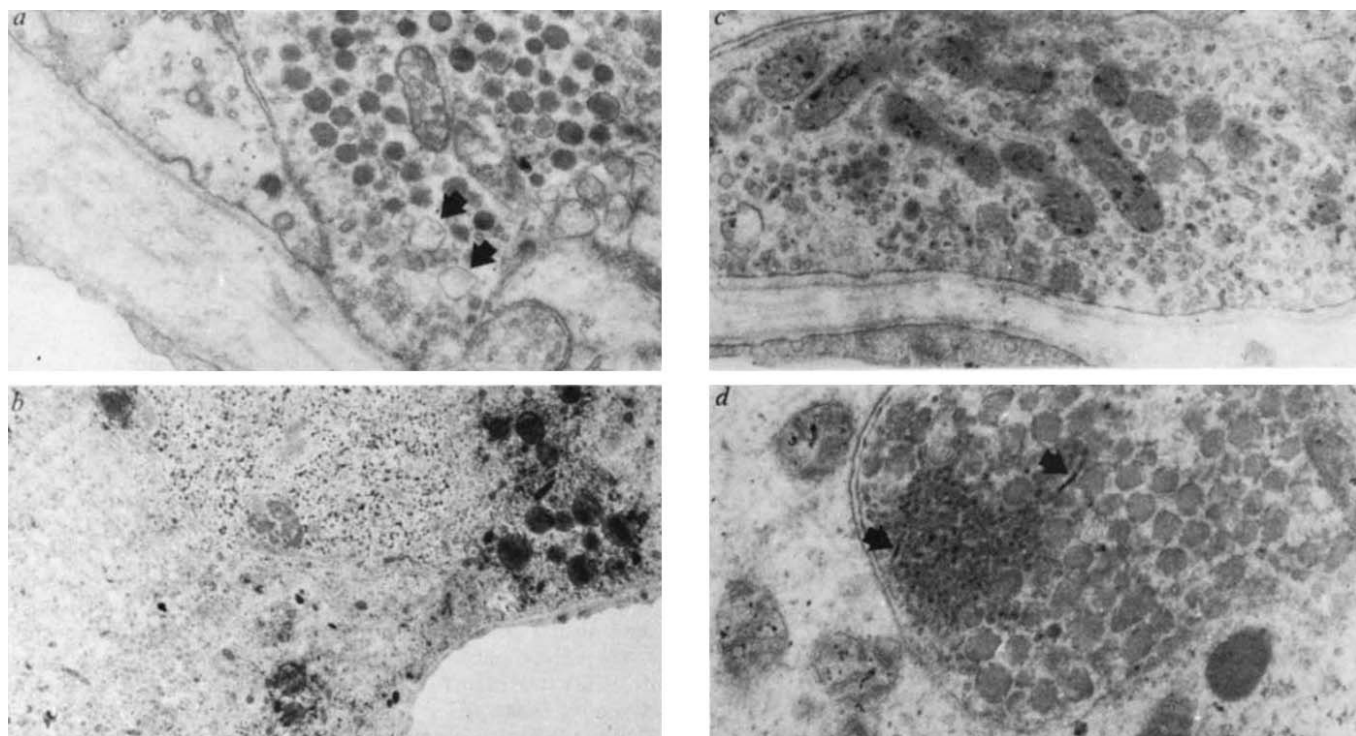


Fig. 1 Electron micrographs of control (*a*) and oxalate/antimonate treated (*b-d*) neurohypophyses. Animals were perfused through an aortic cannula for 10 min with 2.5% glutaraldehyde, 90 mM potassium oxalate, pH 7.2. In some cases, this was preceded by 150 mM potassium phosphate buffer and 3.4% dextran (molecular weight 40,000), pH 7.2, for 1–5 min. The neurohypophyses were dissected out after 16 h and washed for 30 min in 90 mM potassium oxalate, 7.5% sucrose, pH 7.5. The tissue was postfixated for 2 h in 1% osmium tetroxide, 2% potassium antimonate, washed in dilute potassium hydroxide, pH 10, for 15 min, dehydrated rapidly in a graded series of alcohol, and embedded in Spurr resin using propylene oxide. Control tissue was fixed in an identical fashion except that potassium chloride replaced oxalate in the prefix and wash, and 100 mM sodium cacodylate replaced antimonate in the postfix. *a*, Neurosecretory terminal, control method. The terminal lies in contact with a basement membrane, separated from a fenestrated capillary endothelium by a wide perivascular space. It contains neurosecretory granules, mitochondria, and microvesicles, some of which are clustered near the basement membrane contact zone; two vacuoles are also present (arrows; $\times 18,000$). *b*, Low power view of tissue fixed directly with glutaraldehyde/oxalate. The nucleus and cytoplasm of a pituitary (upper region of picture) contain much more precipitate than the surrounding neurosecretory elements; the pituitary cytoplasm also contains characteristic lipid droplets ($\times 2,700$). *c*, Neurosecretory terminal in tissue washed before glutaraldehyde/oxalate fixation. Precipitate is highly localized to mitochondria, microvesicles (in a dispersed configuration here) and a vacuole; it is absent from the perivascular space ($\times 25,000$). *d*, Microvesicles in a clustered formation adjacent to a pituitary in washed tissue. Precipitate is again localized to microvesicles and mitochondria, and also in smooth endoplasmic reticulum associated with the microvesicles (arrows). The pituitary cytoplasm is almost completely free of precipitate ($\times 25,000$).

activity by ionic movements into and through their cytoplasm¹⁴. The observation of calcium precipitates above background level in the pituitary cytoplasm could reflect such a function.

The diffuse cytoplasmic precipitate was rapidly washed out of the neural elements but the mitochondria, vacuoles, smooth endoplasmic reticulum and microvesicles appear to be sites of tightly bound calcium. Intracellular binding sites have been proposed to account for the return of the intracellular ionized calcium level to the very low resting value once stimulation has ceased¹⁵. Localization of calcium in mitochondria was entirely expected in view of their proven calcium-accumulating properties¹⁶, but the available data suggest that the kinetics of brain mitochondrial calcium uptake are not appropriate for dealing with stimulus-induced increases in the level of ionized calcium in nerve terminals¹⁷, when the concentration probably rises from about 10^{-7} to 10^{-5} M at most¹⁸. Mitochondria may well serve as long-term buffers of neuronal intracellular calcium, however¹⁵. Deposits in the vacuoles, which are thought to represent membrane endocytosed after hormone release¹⁹, were also to be expected since they would thereby contain extracellular fluid with a calcium content of about 10^{-3} M.

The precipitates observed in smooth endoplasmic reticulum and microvesicles indicate that these organelles may be sites of calcium accumulation. A non-mitochondrial, ATP-dependent calcium accumulation system has been described in brain

synaptosomes¹⁷, and coated vesicles isolated from cerebral cortex are capable of calcium uptake³. The accompanying letter by Nordmann and Chevallier²⁰ demonstrates that the sub-cellular fractions containing predominantly microvesicles isolated from bovine neurohypophyses can accumulate calcium at concentrations appropriate for the post-stimulus recovery phase. The precipitation pattern described here therefore appears to constitute a morphological correlate of the calcium-accumulating function of microvesicles, which are not associated with any known transmitter. This agrees with evidence that the microvesicles do not alter in number with either acute^{19,21}, or chronic²² stimulation of hormone release and that membrane is retrieved by vacuoles^{19,21} (Fig. 1*a*), rather than by microvesicles as previously suggested²³. The smooth endoplasmic reticulum appears to share this calcium-sequestering property and has been shown to accumulate calcium in synaptosomes²⁴.

The increase in microvesicles labelled with horseradish peroxidase after stimulation²⁵ and the presence of coated microvesicles²³ suggest that these organelles are capable of exocytosis and, indeed, clusters of microvesicles are often seen in 'synaptoid' configurations near the plasmalemma opposite pituitary cells or the basement membrane (Fig. 1*a, c*). Calcium accumulated by microvesicles might therefore be removed from the nerve ending by exocytosis, the dispersed microvesicles near the membrane being active in this process and the clustered

forms (Fig. 1d) representing a less active state. During stimulation, the microvesicles appear to move closer to the contact zone of the ending with the basement membrane¹⁹ and thus their exocytosis might be activated when the gland is stimulated.

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1. Nordmann, J. J., Dreifuss, J. J. & Legros, J. J. *Experientia* **27**, 1344–1345 (1971).
2. Douglas, W. W. & Poinsner, A. J. *Physiol. Lond.* **172**, 19–30 (1964).
3. Blitz, A. L., Fine, R. E. & Toselli, P. A. *J. Cell Biol.* **75**, 135–147 (1977).
4. Borgers, M., de Brabander, M., Van Reempts, J., Anouters, F. & Jacob, W. A. *Lab. Invest.* **37**, 1–8 (1977).
5. Duce, I. R. & Keen, P. *Neuroscience* **3**, 837–868 (1978).
6. Herman, L., Sato, T. & Hales, C. N. *J. ultrastruct. Res.* **42**, 298–311 (1973).
7. Stoekel, M. E., Hindelang-Gertner, C., Dellman, H. D., Porte, A. & Stutinsky, F. *Cell Tissue Res.* **157**, 307–322 (1975).
8. Morris, J. F. *J. Endocr.* **68**, 225–234 (1976).
9. Streit, P. & Reubi, S. C. *Brain Res.* **126**, 530–537 (1977).
10. Klein, R. L., Yen, S.-S. & Thureson-Klein, A. J. *Histochem. Cytochem.* **20**, 65–78 (1972).
11. Birks, R. I. *J. Neurocytol.* **3**, 133–160 (1974).
12. Koch, A., Banck, B. & Neuman, B. L. *Expl. Neurol.* **6**, 186–200 (1962).
13. Dyball, R. E. J. & Shaw, F. D. *J. Physiol., Lond.* **303**, 60–61P (1980).
14. Dellman, H. D., Stoekel, M. E., Porte, A. & Stutinsky, F. *Experientia* **30**, 1220–1221 (1974).
15. Baker, P. F. *Soc. exp. Biol. Symp.* **30**, 67–88 (1976).
16. Lehniger, A. L., Carafoli, E. & Rossi, C. S. *Adv. Enzym.* **29**, 259–320 (1967).
17. Blaustein, M. P., Ratzlaff, R. W., Kendrik, N. C. & Schwartz, E. S. *J. gen. Physiol.* **72**, 14–41 (1978).
18. Baker, P. F. *Sci. Prog. Oxf.* **64**, 95–115 (1977).
19. Morris, J. F. & Nordmann, J. J. *Neuroscience* **5**, 639–649 (1980).
20. Nordmann, J. J. & Chevallier, J. *Nature* **287**, 54–56 (1980).
21. Lescure, H. & Nordmann, J. J. *Neuroscience* **5**, 649–659 (1980).
22. Reinhardt, H. F., Henning, L. C. H. & Rahr, H. P. *Z. Zellforsch. Mikrosk. Anat.* **102**, 182–192 (1969).
23. Nagasawa, J., Douglas, W. W. & Schultz, P. A. *Nature* **332**, 241–242 (1971).
24. McGraw, C. F., Somylo, A. V. & Blaustein, M. P. *J. Cell Biol.* **85**, 228–241 (1980).
25. Theodosis, D. T., Dreifuss, J. J., Harris, M. C. & Orci, L. *J. Cell Biol.* **70**, 294–303 (1976).

Role of corticosteroid-binding globulin in interaction of corticosterone with uterine and brain progesterone receptors

H. Al-Khouri & B. D. Greenstein

Department of Pharmacology, St Thomas's Hospital Medical School, London SE1 7EH, UK

The central actions of the steroid hormone progesterone remain an enigma. However, there is no doubt that this hormone has a vital role in the control of sexual function and behaviour in many species, including man. Furthermore, progesterone may be involved in the premenstrual and postpartum syndromes¹. It would therefore be very useful to know the role and mechanism of action of progesterone in the brain. We now show that there are differences between progesterone receptors in brain and uterus, and possibly in the distribution of the serum progesterone-binding protein, corticosteroid-binding globulin (CBG), which may enter uterine but not brain cells.

Our understanding of the mode of action of steroid hormones was advanced greatly by the discovery of cytosolic receptor proteins which bind the steroid and migrate to the cell nucleus to modify gene expression². Autoradiographic and *in vitro* binding studies have revealed that there are receptors for most classes of steroid hormone in the brain^{3–8}. In this respect, progesterone continues to baffle the investigator and studies of progesterone uptake by the brain remain equivocal^{6,9–12}. The problem is complicated by the extensive metabolism of progesterone in neural tissues¹² and by the presence in rat serum of CBG, which binds both corticosterone and progesterone avidly¹³. A partial solution to the problem was offered by the discovery of large amounts of highly unstable progesterone-binding components

in cytosols from the rat brain, pituitary and uterus^{14,15}. The complex dissociated rapidly *in vitro* even at 0°C, and it was suggested¹⁴ that the rapid dissociation of the complex might account for the failure of previous studies. Recently, a synthetic progesterone, promegestone (R5020, Roussel Uclaf) was introduced; this binds with high affinity to cytosolic proteins in the rat brain, pituitary and uterus^{16–18}. The progesterone receptor seems to meet the requirements for a neural progesterone receptor, as it is increased after oestrogen administration in tissues rich in oestrogen receptors^{17–19}. The progesterone receptor also seems to occur in the cell nucleus^{20,21}. However, several important questions concerning progesterone remain unanswered. Why was the R5020 receptor not discovered using ³H-progesterone? Does R5020 bind to all the progesterone receptors? There is evidence that more than one species of progesterone receptor exists¹⁴. Perhaps most importantly, does progesterone exert its effect in uterus and brain through the same mechanism?

In an attempt to answer some of these questions, cytosols were first prepared from the hypothalamus, midbrain and uterus of adult intact female rats as described previously¹⁴. The rats were perfused with 1 l of chilled normal saline before preparation, after which CBG cannot be detected in brain cytosols using sensitive binding studies¹⁴. After perfusion, the tissues seem normal under light microscopy and the nuclei from these tissues were capable of incorporating ³H-labelled nucleotides into RNA (not shown). The cytosols were then incubated with ³H-progesterone (81 Ci mmol⁻¹, Amersham; 3 × 10⁻⁸ mol l⁻¹) for 2 h at 0°C, when apparent equilibrium between bound and free hormone is attained¹⁴. The ability of a variety of unlabelled steroids (progesterone, corticosterone, R5020, oestradiol-17β and 5-α-dihydrotestosterone (DHT)) to displace specifically bound ³H-progesterone was tested. The experimental procedures are described fully in the legend to Fig. 1, which shows a typical result, and the results of the series of experiments are given in Fig. 2. The results are summarized as follows: (1) in the hypothalamus and midbrain cytosols, progesterone displaced ³H-progesterone far more rapidly and completely than did any of the other steroids tested, including R5020. The nature of the bound radioactive progesterone remaining in midbrain cytosol after displacement with the unlabelled hormone is unclear but may reflect a very slowly dissociating binding component. (2) In the uterus, progesterone and corticosterone were equipotent in displacing rapidly and virtually completely the specifically bound ³H-progesterone. Corticosterone was very much more potent in this respect in the uterus than in the brain tissues. This pattern of displacement of radioactive progesterone from uterine binding sites was also observed if binding isotherms were prepared (Fig. 1 inset). It has been shown elsewhere¹⁴, using binding isotherms, that in brain cytosols corticosterone is a weak inhibitor of ³H-progesterone binding. (3) Oestradiol-17β and DHT were significantly less potent than progesterone in displacing ³H-progesterone from central and peripheral tissues (*P* < 0.001; Student's *t*-test).

The possibility that unlabelled progesterone and corticosterone were displacing ³H-progesterone from plasma CBG, which was not completely flushed from the tissues, was examined by carrying out immunodiffusion studies of cytosols from the rat brain areas, the anterior pituitary and the uterus, using an antibody raised to rat serum. The tissue:buffer ratios and cytosol protein concentrations for the brain areas and uterus were kept as close to each other as possible. Of the tissues tested, only uterine cytosol seemed to react with the antiserum to produce a precipitin line (Fig. 3). Whether the precipitin line shown in Fig. 3 is in fact CBG can only be clarified using the purified protein. The absence of a precipitin line does not mean that no reaction took place using cytosols from the brain, but in view of the equivalence in protein concentrations, this possibility is remote. It is more likely that CBG entered the extravascular compartment in the uterus but not the brain.

Corticosterone caused a total displacement of ³H-progesterone from uterine cytosols. The displacement of ³H-progesterone from uterine cytosols by R5020 must have occurred

Fig. 1 Exchange of ^3H -progesterone and unlabelled progesterone (●), corticosterone (▲) or R5020 (■) in cytosols from the rat brain and uterus. Cytosols were incubated at 0°C with ^3H -progesterone ($3 \times 10^{-8} \text{ mol l}^{-1}$) for 2 h, and the bound radioactivity was assessed by rapid gel filtration of $50 \mu\text{l}$ of the incubate using small columns ($10 \times 0.5 \text{ cm}$) of Sephadex LH20 at 1°C (ref. 14). At time 0 (that is, after 2 h), the unlabelled hormone was added to aliquots of cytosol to give a final concentration of the competitor of $10^{-6} \text{ mol l}^{-1}$ and the bound radioactivity was assessed various times later. Results are shown after correction for nonspecific binding of ^3H -progesterone. For clarity, results using progesterone, corticosterone and R5020 alone are shown (compare with

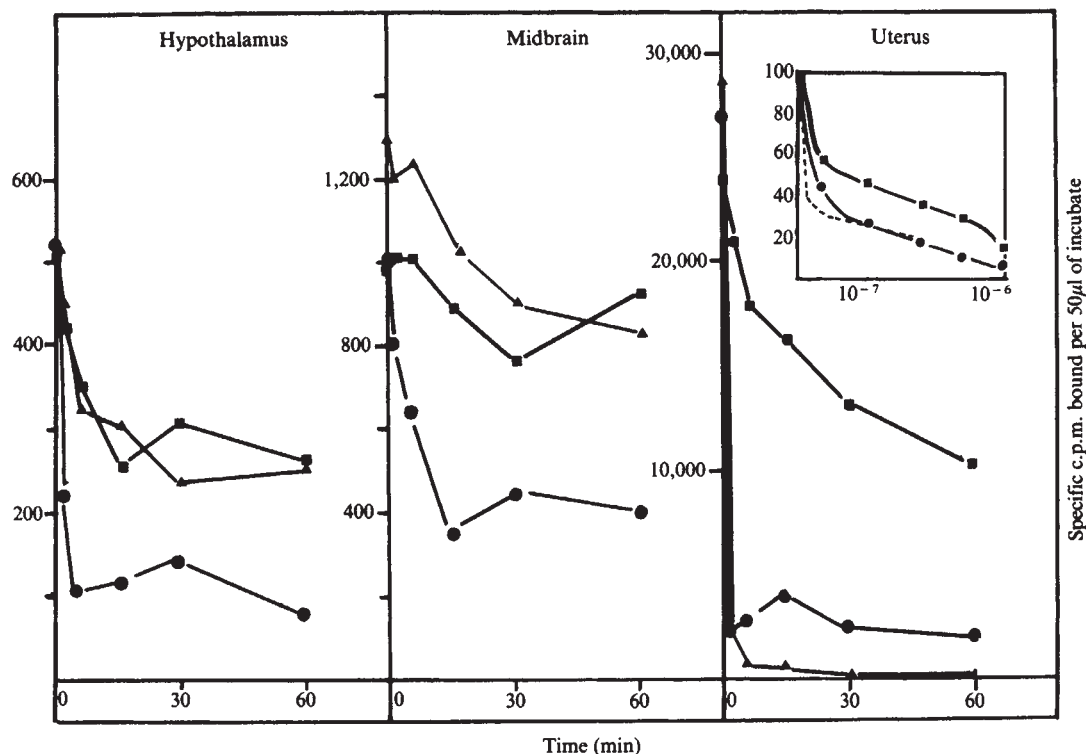


Fig. 2). Abscissa, time after adding unlabelled steroid; ordinate, specific c.p.m. bound per $50 \mu\text{l}$ of incubate. Details of the scintillation spectrometry are given elsewhere¹⁴. Inset: binding isotherms of ^3H -progesterone binding in uterine cytosol in the presence of unlabelled progesterone, R5020 or corticosterone (---). Cytosol was incubated with ^3H -progesterone ($10^{-8} \text{ mol l}^{-1}$) alone or in the presence of varying concentrations of the unlabelled steroid for 2 h at 0°C . Bound radioactivity was assessed as described above. Abscissa, concentration of unlabelled steroid (mol l^{-1}); ordinate, binding of ^3H -progesterone as a percentage of binding in the absence of unlabelled steroid.

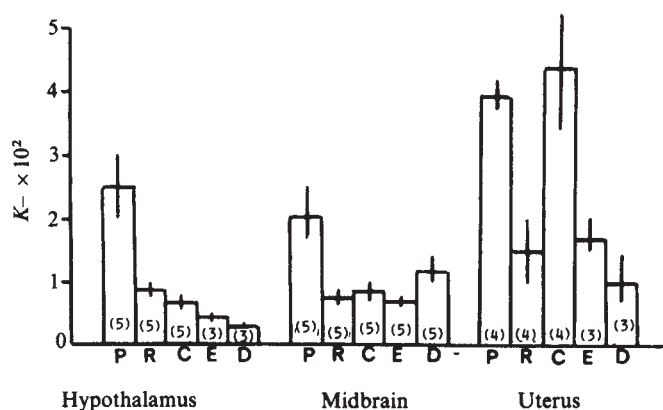


Fig. 2 Displacement of ^3H -progesterone from specific binding sites by unlabelled steroids in hypothalamic, midbrain or uterine cytosols. Results are expressed as the dissociation rate constant (K^-) calculated for each steroid and tissue using the method described in Fig. 1 legend. Results are the mean $\pm$ s.e.m. and the numbers of observations are given in parentheses. K^- was calculated from the formula: $K^- = \ln [(A^0/A)(1/t)]$, where A^0 and A are the specific binding of ^3H -progesterone at times = 0 and 60 min, respectively. P, Progesterone; R, R5020; C, corticosterone; E, oestradiol- 17β ; D, DHT.

that another glucocorticoid, triamcinolone, binds to progesterone receptors in the human prostate²².

The importance of these results lies in the clear demonstration that progesterone, the natural hormone, displaces the tritiated steroid from the receptor in brain and uterine cytosols more potently than does R5020. Furthermore, there may be a

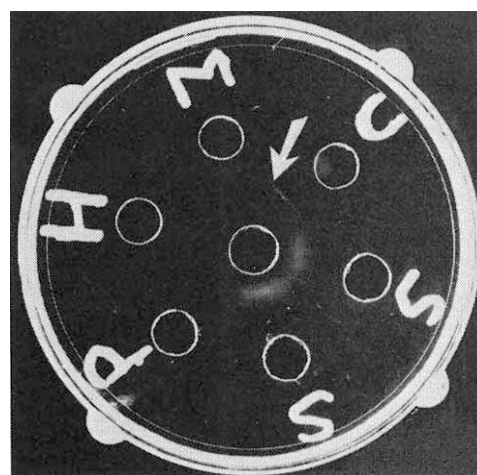


Fig. 3 Immunodiffusion of cytosols from hypothalamus (H), midbrain (M), anterior pituitary (P) and uterine (U) cytosols or rat serum (S) using antiserum raised in a rabbit to whole rat serum (Miles Yeda codes 65-038; lot R134N). To each well $3 \mu\text{l}$ of protein solution was added and this was allowed to diffuse overnight at 20°C . The centre well contained the antiserum. The arrow indicates the precipitin line caused by the reaction between antiserum and uterine cytosol. $6.1 \mu\text{g}$ of midbrain cytosol were applied, $4.5 \mu\text{g}$ of hypothalamic and $5.0 \mu\text{g}$ of uterine cytosol.

exclusively from specific progesterone receptors in the uterus, because this steroid does not bind to CBG¹⁶. If corticosterone had displaced ^3H -progesterone from CBG only, the displacement would have been incomplete. It must be presumed, therefore, that corticosterone does displace ^3H -progesterone from specific progesterone receptors in the uterus, to which it apparently binds much more strongly than in the brain. The functional significance of this is unclear, but there is evidence

selective movement of corticosterone carried by CBG to the extravascular and possibly intracellular compartment in the uterus but not the brain. Thus, CBG could act as a carrier receptor for corticosterone into the uterine cell, where corticosterone displaces progesterone from its receptor site. This action would have important implications in, for example, the onset of parturition. Cortisol is known to initiate parturition in the rabbit and this effect is blocked by the administration of progesterone²³. There are thus grounds for investigating further

the interactions between progesterone, corticosterone and their binding proteins in the blood and uterus with respect to the function of this tissue in reproduction.

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1. Dalton, K. *The Premenstrual Syndrome* (Heinemann, London, 1974).
2. King, R. J. B. & Mainwaring, W. I. P. *Steroid-Cell Interactions* (Butterworths, London, 1974).
3. Stumpf, W. E. *Science* **162**, 1001-1003, (1968).
4. Pfaff, D. W. *Science* **161**, 1355-1356 (1968).
5. Kato, J. & Onouchi, T. *Endocr. jap.* **20**, 429-432 (1973).
6. McEwen, B. S., de Kloet, R. & Wallach, G. *Brain Res.* **105**, 129-136 (1976).
7. Ginsburg, M., Greenstein, B. D., MacLusky, N. J., Morris, I. D. & Thomas, P. J. *Steroids* **23**, 773-792 (1974).
8. Barley, J., Ginsburg, M., Greenstein, B. D., MacLusky, N. J. & Thomas, P. J. *Brain Res.* **100**, 383-393 (1975).
9. Wade, G. N. & Feder, H. H. *Brain Res.* **45**, 525-543 (1972).
10. Lutge, W. C., Wallis, C. J. & Hall, N. R. *Brain Res.* **71**, 105-115 (1974).
11. Sar, M. & Stumpf, W. E. *Science* **182**, 1266-1268 (1973).
12. Karavolas, H. J., Hodges, D. R., O'Brien, D. J. & McKenzie, K. M. *Endocrinology* **104**, 1418-1425 (1979).
13. Westphal, U. in *Biochemical Actions of Hormones* Vol. 1 (ed. Litwack, G.) 209-265 (Academic, New York, 1972).
14. Greenstein, B. D. *J. Endocr.* **79**, 327-338 (1978).
15. Lee, H., Davies, I. J. & Ryan, K. J. *Endocrinology* **104**, 791-800 (1979).
16. Moguilewsky, M. & Raynaud, J.-P. *Steroids* **30**, 99-109, (1977).
17. Kato, J. & Onouchi, T. *Endocrinology* **101**, 920-928 (1977).
18. MacLusky, N. J. & McEwen, B. S. *Nature* **274**, 276-278 (1978).
19. Moguilewsky, M. & Raynaud, J.-P. *Endocrinology* **105**, 516-522, (1979).
20. Blaustein, J. D. & Wade, G. N. *Brain Res.* **140**, 360-367 (1978).
21. Seiki, K., Haurki, Y., Imanishi, Y. & Enomoto, T. *J. Endocr.* **82**, 347-360 (1979).
22. Zava, D. T., Landrum, B., Horwitz, K. B. & McGuire, W. L. *Endocrinology* **104**, 1007-1012 (1979).
23. Nathaniel, P. W. & Abel, M. J. *J. Endocr.* **57**, 47-54 (1973).

Action of formamidine pesticides on octopamine receptors

Peter D. Evans

ARC Unit of Invertebrate Chemistry and Physiology, Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK

Julian D. Gee

Pfizer Central Research, Pfizer Limited, Sandwich, Kent CT13 9NJ, UK

The formamidines are a structurally novel group of pesticides of growing importance in the control of mites, cattle ticks and certain orders of insects which have become resistant to conventional acaricides and insecticides. Their mode of action is complex with dose-dependent lethal and sublethal effects¹⁻³. At sublethal levels they cause behavioural changes in the target pest species (for example in feeding and in mating behaviours), changes which are responsible for the protective effects on crops and livestock. Although many suggestions have been made for the underlying biochemical mechanism, including inhibition of monoamine oxidase activity, uncoupling of respiration and blockade of neuromuscular transmission¹, no direct evidence has been presented. Another possibility is interaction with octopamine receptors in the central nervous system^{2,4,5}. We report here that the formamidine acaricide/insecticide, chlordimeform (CDM), and its demethylated derivative can mimic the actions of octopamine at the locust neuromuscular junction. This gives the clearest evidence to date of the site of action of the formamidines and indicates a novel mode of action for these pesticides.

Octopamine is one of several compounds that mimic the action of CDM in exciting certain unidentified neurones in the abdominal ganglia of the larvae of the tobacco hornworm, *Manduca sexta*. The activation of these neurones may be correlated with the increased locomotor activity and uncoordination produced in these animals by sublethal doses of CDM². Octopamine, as well as CDM and some of its derivatives elicit glowing⁶, and increase adenylate cyclase activity⁷ in the firefly light organ^{2,4,5}. Octopamine may be a neurotransmitter in the firefly light organ but in both cases definitive proof for octopaminergic neurones is lacking. However, an identified octopaminergic neurone has been shown to act at the neuromuscular

junction of the locust, *Schistocerca americana gregaria*^{8,9,10} and the pharmacology of the octopamine receptors has been investigated¹¹. This preparation can thus provide a model system in which to study the interaction of the formamidines with identified octopamine receptors.

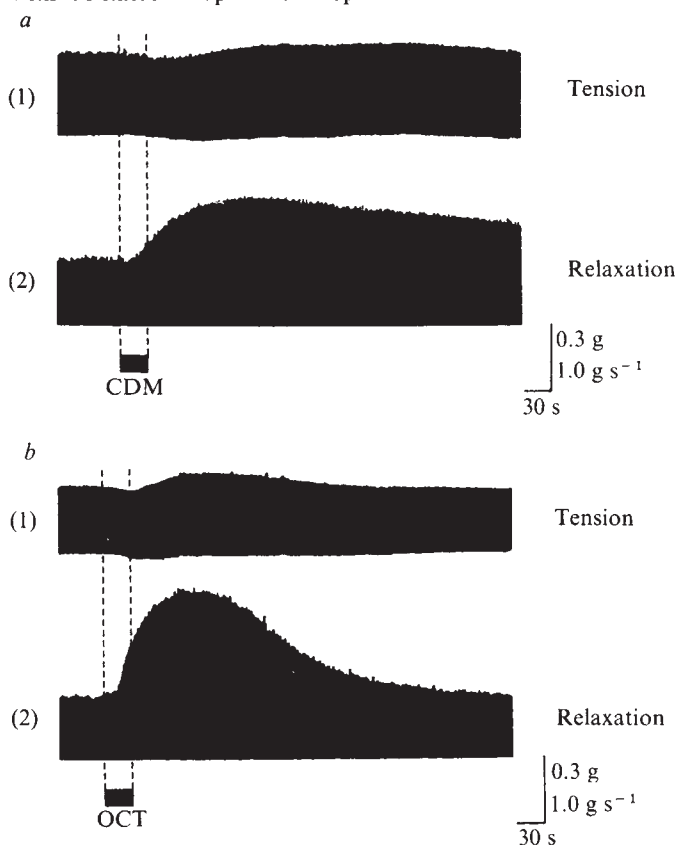
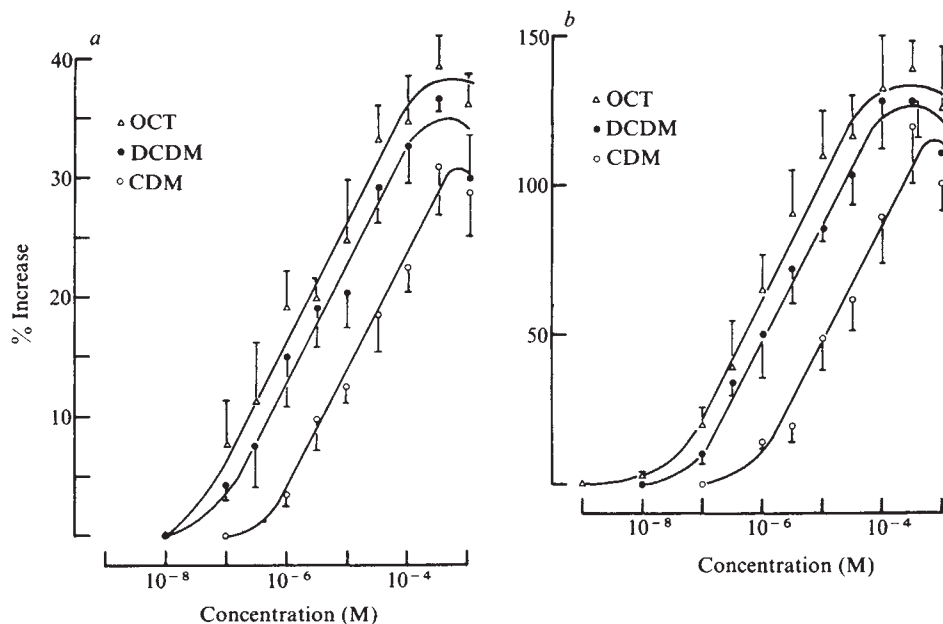


Fig. 1 Comparison of the effects of chlordimeform (CDM) (a) and ($\pm$)octopamine (OCT) (b) on the amplitude of twitch tension and rate of relaxation of twitch tension induced in the extensor tibiae muscle of the hindleg of the locust by stimulation of the slow motoneurone at 1 Hz. Traces a1 and b1 show the tension responses elicited by application of a 30 s pulse of 10 μ M chlordimeform and ($\pm$)octopamine respectively. Traces a2 and b2 show the effects of the respective pulses on the rate of relaxation of twitch tension, the height of each trace being directly proportional to the rate of relaxation. Note the difference in potency and time course of the effects produced by chlordimeform and octopamine.

Fig. 2 Dose-response curves for the potentiation of twitch amplitude (a) and relaxation rate (b) by octopamine (OCT), chlordimeform (CDM) and demethylchlordimeform (DCDM). The results represent the maximum percentage increases obtained at each concentration by application of a 30 s pulse of drug solution to an extensor-tibiae muscle preparation being stimulated at 1 Hz. Each point represents the mean of at least four determinations and the bars represent the s.e.m.



We stimulated repetitively the slow motoneurone (SETi) to the extensor tibiae muscle of the hindleg of *S. americana gregaria* to elicit individual muscle twitches. Using methods described previously, we continuously monitored the amplitude of muscle twitches^{8,10} and their rates of contraction and relaxation¹². Short pulses of known concentrations of the formamidines were introduced into the superfusion fluid bathing the muscle preparation and their effects compared to those produced by comparable pulses of ($\pm$)octopamine.

Figure 1 compares the effects produced by a 30 s pulse of 10 μ M CDM (Fig. 1a) with those produced by a similar pulse of ($\pm$)octopamine (Fig. 1b). CDM potentiates the size of neurally evoked contractions by 10% and increases the rate of relaxation by 91%. The equivalent pulse of octopamine is more potent and increases the two parameters by 23% and 166% respectively. The time course of the effects of the two compounds differs, the effect of CDM on the relaxation rate, for example, having a half time of decay of 6.7 min whilst that for octopamine is 1.8 min in the particular examples illustrated.

A comparison of the dose-response curves for octopamine, CDM and demethylchlordimeform (DCDM) (Fig. 2a, b) reveals that the curves are parallel with peak values at 10^{-4} to 10^{-3} M for both the potentiation of twitch amplitude and the increase in relaxation rates. There is no significant difference between the curves for octopamine and DCDM and, the effects of these two compounds at saturating concentrations were not additive (data not shown). As octopamine is the only naturally occurring compound known to be capable of producing these modulatory effects at the locust neuromuscular junction¹⁰ it is likely that CDM and octopamine are acting at the same receptor. Comparing CDM with DCDM (to which CDM is thought to be metabolized to give the lethal toxicant in CDM-poisoned cattle tick larvae¹³) half maximal percentage increases of both twitch amplitude and relaxation rate are obtained at 5×10^{-6} M for DCDM and 5×10^{-5} M for CDM. Thus DCDM is an order of magnitude more effective than CDM. This is in agreement with the rank order of potency of the phenolamines on this preparation where synephrine (the *N*-methylated analogue of octopamine) is more potent than *N,N*-dimethyloctopamine¹⁰. The structural similarities between the formamidines and the phenolamines have been pointed out and the close resemblance between DCDM and synephrine emphasized⁴.

Octopamine receptors in insects are selectively blocked by α -adrenergic antagonists, such as phentolamine, in preference to β -adrenergic antagonists such as propranolol^{7,9,10,11}. Phentolamine also selectively antagonises the action of CDM at the locust neuromuscular junction. Figure 3 shows an experiment

where the effects of 10 μ M ($\pm$)propranolol and 10 μ M phentolamine are compared after an initial potentiation of twitch tension and relaxation rates by exposure of the extensor muscle to a 30 s pulse of 10 μ M CDM. Propranolol has no effect, whereas phentolamine brings about a rapid reduction in both responses. Other experiments (not shown) demonstrate that phentolamine, but not propranolol, blocks the effects of CDM applied simultaneously to the extensor muscle.

We have demonstrated that both CDM and DCDM can mimic the actions of octopamine in potentiating twitch tension and increasing the rate of relaxation of tension induced in the extensor tibiae muscle. In both cases the effects are brought about by an interaction with pharmacologically characterized octopamine receptors¹¹. In addition to its effects on neurally evoked tension, octopamine inhibits a myogenic rhythm of contraction and relaxation found in a bundle of specialized muscle fibres in the extensor tibiae muscle of the locust hindleg^{9,14}. This effect is also mimicked by CDM and DCDM (not shown). The frequency of the myogenic contractions is reduced by 50% by exposure to 10^{-6} M CDM for 10 min.

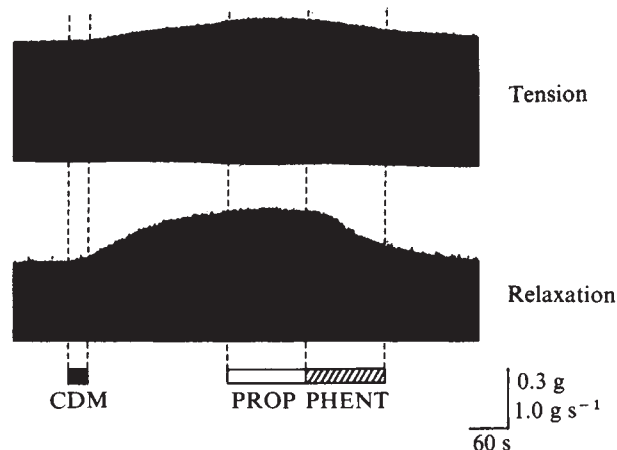


Fig. 3 The effect of blocking agents on the responses of the extensor tibiae muscle to a 30 s pulse of 10 μ M chlordimeform (CDM). The top trace indicates the tension responses recorded from the muscle by stimulating the slow motoneurone at 1 Hz and the bottom trace shows the corresponding changes in relaxation rates. ($\pm$)propranolol (PROP) 10 μ M was applied for a 2-min period indicated by the open bar and 10 μ M phentolamine (PHENT) for a 2-min period indicated by the hatched bar.

The effects of the formamidines on neuromuscular transmission in the locust parallel those on the firefly light organ in several respects. In both cases DCDM is more effective than CDM, and their actions are blocked by phentolamine⁴. In the studies on the firefly light organ, however, the exact concentration of the formamidines superfused over the preparation was not reported and the effects of α - and β -adrenergic blocking agents were not compared. The effects of the formamidines are also much longer lasting than those of octopamine in both the firefly and locust preparations. It has been hypothesized that this might be due to a greater tissue solubility of the formamidines which would slow down their penetration to the receptors and maintain an excitatory concentration of the compounds close to the receptors for a longer time than an equivalent dose of octopamine⁴. The longer half-time of decay of the effects of CDM, however, suggests that the formamidines have a longer half-time of receptor occupancy than octopamine.

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1. Beeman, R. W. & Matsumura, F. in *Pesticide and Venom Neurotoxicity* (eds Shankland, D. L., Hollingworth, R. M. & Smyth, T. Jr) 179–188 (Plenum, New York, 1978).
2. Lund, A. E., Hollingworth, R. M. & Murdock, L. L. in *Advances in Pesticide Science*, Part 3 (ed. Geissbuehler, H.) 465–469 (Pergamon, Oxford, 1979).
3. Lund, A. E., Hollingworth, R. M. & Shankland, D. L. *Pest. Biochem. Physiol.* **11**, 117–128, (1979).
4. Murdock, L. L. & Hollingworth, R. M. in *Insect Neurobiology and Pesticide Action*, 415–422 (Soc. Chem. Ind., London, 1980).
5. Hollingworth, R. M. & Murdock, L. L. *Science* **208**, 74–76 (1980).
6. Carlson, A. D. *J. exp. Biol.* **49**, 195–199 (1968).
7. Nathanson, J. A. *Science* **203**, 65–68 (1979).
8. Evans, P. D. & O'Shea, M. *Nature* **270**, 275–279 (1977).
9. Evans, P. D. & O'Shea, M. *J. exp. Biol.* **73**, 235–260 (1978).
10. O'Shea, M. & Evans, P. D. *J. exp. Biol.* **79**, 169–190 (1979).
11. Evans, P. D. in *Receptors for Neurotransmitters, Hormones and Pheromones in Insects* (eds Sattelle, D. B., Hall, L. M. & Hildebrand, J. G.) 147–158 (Elsevier, Amsterdam, 1980).
12. Buchan, P. B. & Evans, P. D. *J. exp. Biol.* **85**, 349–352 (1980).
13. Knowles, C. O. & Schuntner, C. A. *J. Aust. ent. Soc.* **13**, 11–16 (1974).
14. Hoyle, G. *J. exp. Zool.* **193**, 425–431 (1975).

The sodium channel and intracellular H⁺ blockage in squid axons

Enzo Wanke, Emilio Carbone & Pier Luigi Testa

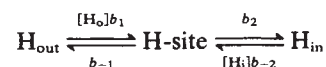
Laboratorio di Cibernetica e Biofisica del CNR,
16032-Camogli (GE), Italy

Sodium channels in plasma membranes can be blocked by a large variety of toxins¹ and local anaesthetics². This property, however, is not confined to relatively large molecules. For instance, extracellularly applied small ions like hydrogen may also prevent the passive transport of permeant cations across open Na⁺ channels^{3–6}. A typical feature of this phenomenon^{3–6} is that the blocking action of hydrogen is gradually relieved by increasing the voltage applied across the membrane. Although in the frog skeletal muscle there is no clear evidence for a similar intracellular action⁷, we report here for the squid giant axon remarkable effects on the ionic permeability of Na⁺ channels when the internal perfusate contains an excess of protons. Analysing the action of low pH inside and outside the fibre in terms of a kinetic model³, we could conclude that Na⁺ channels in squid axons are controlled by two independent groups: one with an apparent pK_a of 4.6 and the other with pK_a 5.8, the former feeling one-fifth of the applied membrane potential, the latter three-quarters. As with pharmacological agents⁸, we also show that the voltage-dependence of the H⁺ blockage is not affected by the presence of the inactivation gate.

Figure 1 shows computer records of Na conductance at normal and low intracellular pH (pH_i) obtained from giant axons of the squid *Loligo vulgaris* perfused and voltage-clamped by the method of Wanke *et al.*⁹. The traces on the right were recorded after 90 s of internal perfusion at pH_i 5.3 and were found to reverse by returning to normal conditions (pH_i 7.2). Applications of low pH_i produce remarkable alterations to both the time course and voltage-dependence of the Na⁺-channel conductance. The peak conductance, g_{Na}^P , is strongly depressed below 60 mV while at higher depolarizations the amount of depression is gradually reduced by raising the voltage (Fig. 2a). Significantly, at 220 mV the difference of g_{Na}^P at pH_i 7.2 and 5.3 is less than 20% of the control, with a tendency to diminish further at higher potentials. As shown in Fig. 2a, lower pH_i has even more marked effects on the shape of $g_{Na}^P(E)$, supporting the idea that the block of g_{Na} by hydrogen ions follows the titration property of an acidic sidegroup. Peak conductance relative to the control at $E = 100$ mV is plotted in Fig. 2d.

Figure 1 gives a clear indication that the voltage-dependence of the H⁺ block is not related to the action that pH_i has on the gating of both the activation and inactivation processes¹⁰. This view is supported by the observation that at the end of the g_{Na} records ($t = 4$ ms), when fast Na⁺ inactivation is almost complete, the change in g_{Na} with increasing voltage parallels that of g_{Na}^P in the range 140–220 mV. A direct confirmation came from a series of experiments in which the effects of low pH_i were tested in axons treated with the enzyme mixture Pronase¹¹, which removes selectively the fast inactivation gate of Na⁺ channels. As expected, in four trials the effects were similar to those with intact axons (Fig. 2b). Several other controls were done to verify that the H⁺ blockage was genuine. On a total of more than 30 axons, replacements of the internal ionic composition, type and amount of buffers yielded results analogous to those in Fig. 1.

Although a rigorous treatment of the present data would have required the use of a complete four-barrier model for the Na⁺ channel¹², we found it more profitable in our preliminary studies to use the simpler two-barrier model proposed by Woodhull³. The model postulates the existence of a receptor site for H⁺ ions located part of the way across the Na⁺ channel, having the following properties: (1) the rates of hydrogen binding and unbinding are exponential functions of voltage as in Eyring rate theory; (2) an H⁺ ion can reach the site from either side of the membrane according to the scheme



where b_i indicate the voltage-independent components of the

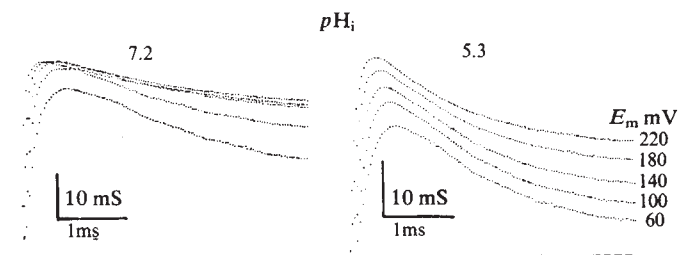


Fig. 1 Time course of Na conductance at neutral (left) and acid (right) pH_i . The membrane was hyperpolarized to -90 mV for 180 s to remove slow inactivation and then depolarized to the test potential indicated. Successive stimuli were separated by 1 s. Positive feedback was used. Data were obtained from current records corrected for TTX-insensitive currents after division by the driving force $E - E_{Na}$. E_{Na} was -26 mV. During stimulation, the periaxonal accumulation of Na⁺ ions was controlled by brief hyperpolarizations⁹. In this way E_{Na} was found to increase by less than 3 mV. Internal solutions (mM): 200 Na⁺, 117 F⁻ and H₂PO₄⁻ (pH_i 7.2) or 200 Na⁺, 80 F⁻ and 45 citric acid (pH_i 5.3). External bath (mM): 109 Na⁺, 326 tetramethylamine, 10 K⁺, 20 Tris, 10 Ca²⁺, 40 Mg²⁺, 555 Cl⁻ (pH_o 8). Temperature 2 °C.

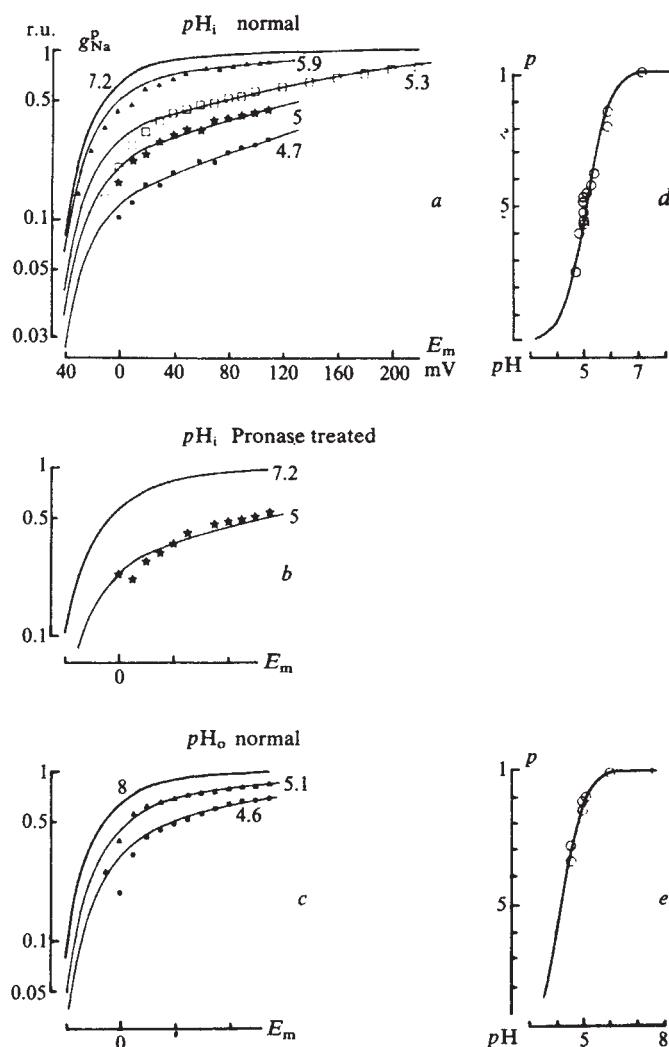


Fig. 2 Action of low pH_i on peak sodium conductance, $g_{Na}^P(E)$. *a*, *b* and *c*, symbols indicate experimental data. Solid lines at control pH represent an average from seven axons at pH_i 7.2 and pH_o 8. Continuous lines at test pH are the product of the control curves and the fraction of unblocked channels, p , as derived by Woodhull³:

$$\left[1 + \frac{[H_o] + [H_i]b_{-2}/b_1 \exp FE/2RT}{b_{-1}/b_1 \exp \delta FE/RT(1 + b_{-2}/b_1 \exp -FE/2RT)} \right]^{-1} \quad (1)$$

where δ is the fraction of the potential drop crossed on approaching the site from the outside; b_i are rate constants (see text) and F , R , T have their usual meaning. Solid lines in *c* at pH_o 5.1 and 4.6 were obtained by setting in equation (1): $\delta = 0.20$, $b_{-2}/b_1 = 0.01$ and $b_{-1}/b_1 = 2.4 \times 10^{-5}$. Corrected data and standard solutions at pH_i 7.2 and pH_o 8 are the same as those of Fig. 1. Internal solutions of low pH all contained 200 mM Na^+ and 45 mM of the following buffers: $H_2PO_4^-$ (pH_i 5.9), citric (5.3, 5 and 4.7). External solutions of low pH had the same ionic content as in Fig. 1 except for the buffer which was 20 mM citric acid for pH_o 5.1 and 4.6. The pronase-treated fibre was perfused for 100 s with a solution containing 2 mg ml^{-1} of enzyme at pH_i 9.9. *d*, *e*, Internal and external pH dependence of the quantity p at $E_m = 100$ mV. Each dot represents a different axon. Solid lines through data points are derived from equation (1) by setting $E_m = 100$ mV.

rate constants and $[H_i]$, $[H_o]$ the concentrations of protons inside and outside the fibre.

As shown in Fig. 2, the model allows an evaluation of the fraction of unblocked channels, p , in terms of three independent parameters: δ , b_{-2}/b_1 and b_1/b_{-1} (see Fig. 2 legend). For the internal block, good fits of the g_{Na}^P curves at low pH_i were obtained by setting: $\delta = 0.75$, $b_{-2}/b_1 = 1$ and $b_1/b_{-1} =$

1.6×10^{-6} . Few points at low depolarizations are systematically lower than the theoretical predictions, suggesting the existence of a small unexpected shift of $g_{Na}^P(E)$ toward depolarizing voltages. Interestingly, with the same set of parameters we could also fit the pH dependence of the block shown in Fig. 2*d*.

With the view of testing further the validity of the Woodhull's model in squid axons, we reinvestigated the action of low extracellular pH (ref. 13), pH_o , on g_{Na}^P . As shown in Fig. 2*c*, *e*, low pH_o is apparently less effective in blocking open Na^+ channels than a corresponding low pH_i value, suggesting the existence of different titratable groups on each side of the channel: one external with an apparent pK_a of 4.6 and one internal with pK_a 5.8. It is also evident from Fig. 2 that the reduction of g_{Na}^P at pH_o 4.6 is smaller than that reported by Carbone *et al.* for unperfused squid axons (probably due to the present improved pH_i control; compare Fig. 3*b* in ref. 13). Lowering the pH of the external bath is known to decrease the intracellular pH (ref. 14). Thus, it is very likely that in intact fibres the effects of low pH_o superpose partly on those of low pH_i , implying that axons with uncontrolled pH_i might be more sensitive to changes of pH_o . Although in frog nodes³ and skeletal muscles⁵ the apparent pK_a of the blocking site is systematically higher than the value found here (5.3 against 4.6 for perfused axons), our results indicate a substantial similarity in the external properties of Na^+ channels for the three types of membranes.

The presence of an acid group inside the channel accessible from the axoplasmic region is in good agreement with the finding that other pharmacological agents, such as pancuronium¹⁵ and 9-aminoacridine⁸ (9-AA), affect open Na^+ channels of squid axons in a voltage-dependent manner. Both molecules are positively charged at pH_i 7.2 and have a sufficiently high affinity for their binding site ($K_a = 6.2 \times 10^{-5}$ M and $\delta = 0.5$ for 9-AA) to suggest that the acid group in question could be identified with part of their receptor site. Experiments concerning the pH dependence of their rate of action would certainly clarify this theory. Interestingly, the site is hardly accessible to high concentrations of tetrodotoxin (TTX)¹⁶ even when the toxin is applied intracellularly at various pH_i values (own observation). This promptly suggests that the molecular arrays surrounding the internal and the external acid groups might be remarkably different.

To our knowledge there are no other reports indicating the existence of an intracellularly negative group within the Na^+ channel. Recently, Nonner *et al.*⁷ have shown that in the frog skeletal muscle low pH_i does not produce significant reductions of the peak sodium permeability. They also showed that some inactivation-removing agents previously tested in squid axons have different effects when applied to the muscle membrane. This indicates that we should not make generalizations based on the present findings and suggests that although Na^+ channels of various plasma membranes have similar external regions, they might differ substantially in the unexposed internal part.

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- Narahashi, T. *Physiol. Rev.* **54**, 813-889 (1974).
- Hille, B. *J. gen. Physiol.* **69**, 475-496 (1977).
- Woodhull, A. M. *J. gen. Physiol.* **61**, 687-708 (1973).
- Schauf, C. F. & Davis, F. A. *J. gen. Physiol.* **67**, 185-195 (1976).
- Campbell, D. T. & Hille, B. *J. gen. Physiol.* **67**, 309-323 (1976).
- Ohmori, H. & Yoshii, M. *J. Physiol., Lond.* **267**, 429-463 (1977).
- Nonner, W., Spalding, B. C. & Hille, B. *Nature* **284**, 360-363 (1980).
- Yeh, J. Z. *J. gen. Physiol.* **73**, 1-21 (1979).
- Wanke, E., Carbone, E. & Testa, P. L. *Biophys. J.* **26**, 319-324 (1979).
- Carbone, E., Testa, P. L. & Wanke, E. *Biophys. J.* (submitted).
- Armstrong, C. M., Bezanilla, F. & Rojas, E. *J. gen. Physiol.* **62**, 375-391 (1973).
- Hille, B. *J. gen. Physiol.* **66**, 535-560 (1975).
- Carbone, E., Fioravanti, R., Prestipino, G. & Wanke, E. *J. Membrane Biol.* **43**, 295-315 (1978).
- Bicher, H. I. & Ohki, S. *Biochim. biophys. Acta* **255**, 900-904 (1972).
- Yeh, J. Z. & Narahashi, T. *J. gen. Physiol.* **69**, 293-323 (1977).
- Rojas, E. & Rudy, B. *J. Physiol., Lond.* **262**, 501-531 (1976).

Solubilization of the choline transport system and re-incorporation into artificial membranes

R. G. King & R. M. Marchbanks

Department of Biochemistry, Institute of Psychiatry, London SE5 8AF, UK

The carrier-mediated transport of choline has been described in several types of cell¹⁻³ and at isolated synaptic terminals⁴. The operation of the carrier seems to be a rate-controlling factor for the synthesis of acetylcholine at the synaptic terminals that release this neurotransmitter⁵. The molecular mechanism by which the carrier transports choline is unknown and difficult to investigate because at present it can only be studied in the complex environment of the natural membrane in which it is situated. Furthermore, such studies are hampered by the results of metabolic activity inside the cell itself. The resolution of these difficulties by the separation, purification and examination of carriers in defined systems is hindered by the problem of assaying its activity during the purification procedure. We describe here the separation of the choline carrier from the membrane and its re-incorporation into unilamellar liposomes with retention of its functional activity, thus enabling its purification and examination in isolation to be undertaken.

The starting material used was synaptic plasma membrane of guinea pig cerebral cortex produced from hypo-osmotically shocked synaptosomes (nerve ending particles) by the procedure of Jones and Matus⁶. Membrane material was solubilized with cholate using a modification of the procedure of Kagawa and Racker⁷, but as in Racker's original method⁸, no urea was present, and 1 mM dithiothreitol was added. Insoluble material was removed by centrifugation at 100,000g for 30 min.

A mixture of purified lipids of approximately similar composition to synaptic plasma membrane⁹ (40% phosphatidylcholine, 30% phosphatidylethanolamine, 20% cholesterol, 10% phosphatidyl serine w/w) were dispersed by ultrasonication, added to the cholate extract (3 mg ml⁻¹ lipids, 2 mg ml⁻¹ protein) and dialysed⁷. As dialysis proceeds, unilamellar liposomes are formed and these incorporate proteins and lipids from the original cholate extract. Choline transport was measured by observing the uptake of ³H-choline, the liposomes having been separated from the incubation medium by Millipore filtration followed by two washes.

As shown in Fig. 1, ³H-choline uptake by the liposomes is reduced by the presence of saturating concentrations (10 mM) of nonradioactive choline chloride during the first 4 min of uptake: this effect is not seen after periods of 30 min or more. A comparison with the uptake into liposomes prepared from pure lipid alone cannot be made on a protein basis, but the ratio can be expressed as the fraction of equilibration value or on a phospholipid basis. As Table 1 shows, it is the incorporation of membrane extract into the liposomes that increases the rate of choline uptake and confers saturability. Hemicholinium had no statistically significant effect on pure lipid liposomes.

To guard against the possibility that ³H-choline is bound to the liposomes, 10 mM choline was present in the washing solution in all experiments. A further check is that trans-activation of influx can be demonstrated in the protein-loaded liposomes. It has been shown in many transport systems, including that for choline^{3,5}, that preloading with the transported substance actually increases the uptake of that substance. If binding were responsible for the results of Fig. 1, preloading the liposomes with choline chloride would be expected to diminish ³H-choline uptake. However, as shown in Table 2, preloading

Table 1 Comparison of ³H-choline uptake by pure lipid liposomes and liposomes made with lipid and cholate-solubilized extract of synaptic plasma membrane

	Fraction of equilibrated value (±s.e.m.(n))	
	In 4 min	In 60 min
Lipid alone	0.26 ± 0.05(8)	0.79 ± 0.09(8)
Lipid alone + 10 mM choline chloride	0.30 ± 0.04(8)	0.80 ± 0.12(8)
Lipid + extract of s.p.m.	0.49 ± 0.05(6)	0.78 ± 0.08(6)
Lipid + extract of s.p.m. + 10 mM choline chloride	0.32 ± 0.03(6)	0.75 ± 0.10(6)

Liposomes were prepared as described in the text, and uptake experiments carried out as described in Fig. 1 legend. Pure lipid liposomes were prepared in the same way with cholate but without the addition of cholate-solubilized extract of synaptic plasma membrane (s.p.m.). Phospholipid content of the liposomes was measured, and the initial rate of uptake expressed on a basis of phospholipid content was 0.46 pmol per mg phospholipid per min (lipid and extract of synaptic plasma membrane) and 0.20 pmol per mg phospholipid per min (lipid alone); both measurements were made at a choline concentration of 1 µM.

with cold choline caused completely the reverse. The demonstration in Table 2 that influx is increased by preloading with choline also shows that an important feature of the *in vivo* transport of choline is retained in the artificial membrane system.

Hemicholinium-3 is a specific inhibitor of choline transport¹⁰. It has an inhibitory effect (Table 2) on choline uptake in protein-containing liposomes, although we found some variability in its effectiveness. Choline transport is known to be sensitive to the Na⁺ and K⁺ gradients across the membrane^{3,4}. Whether or not this is due to a requirement for sodium co-transport, an inwardly directed negative electrical gradient, or some other mechanism, is unknown. Liposomes were prepared in a predominantly KCl medium and then transferred to a larger volume of predominantly NaCl medium so as to mimic the ion gradient found normally. It was found that liposomes prepared in KCl had

Table 2 Effects of hemicholinium, preloaded choline and valinomycin on uptake of ³H-choline by liposomes prepared with cholate extract of synaptic plasma membrane

	Initial rate as % control ± s.e.m.(n)
Liposomes + 10 mM choline chloride	7 ± 12(8)
Liposomes + 100 µM hemicholinium	72 ± 10(8)
Liposomes preloaded with 2 µM choline chloride	132 ± 16(8)
Liposomes preloaded with 10 µM choline chloride	192 ± 24(8)
Liposomes preloaded with 15 µM choline chloride	208 ± 32(8)
Liposomes made in K ⁺ medium and then transferred to Na ⁺ medium:	
Liposomes + 10 mM choline chloride	52 ± 8(10)
Liposomes + 8 µM valinomycin	155 ± 22(10)
Liposomes + 8 µM valinomycin + 10 mM choline chloride	104 ± 18(10)

Liposomes were prepared as described in the text (those made in K⁺ medium were prepared in the same way, except that 100 mM KCl replaced 100 mM NaCl in all solutions). Uptake over 30 s was measured as in Fig. 1, except that the liposomes were added to a larger volume (360 µl) of NaCl-based dialysis medium containing 2–3 µCi ³H-choline. Preloading was by incubation with choline chloride until equilibrium was reached (control liposomes incubated as well). External choline concentration during ³H-choline uptake was 5 µM (except where 10 mM choline was present). The two control groups of liposomes were in the medium as described above, but with none of the additions, and the rates of uptake were: 1.9 pmol per mg protein per min (upper part) and 3.0 pmol per mg protein per min (lower part), both at a choline concentration of 5 µM.

approximately 50% greater initial rate of uptake of ^3H -choline than liposomes prepared in a predominantly NaCl medium. Also, valinomycin (which could be expected to produce a potential across the membrane by specifically increasing its permeability to K^+) caused an increased uptake of ^3H -choline by the liposomes; this, however, was not inhibited by 10 mM choline chloride. The presence of certain other substances in the external solution, including 2 mM MgCl_2 , 1 mM CaCl_2 and 3 mM MgATP , had no statistically significant effect; however, we have not tested the effects of different concentrations of these substances inside the liposomes.

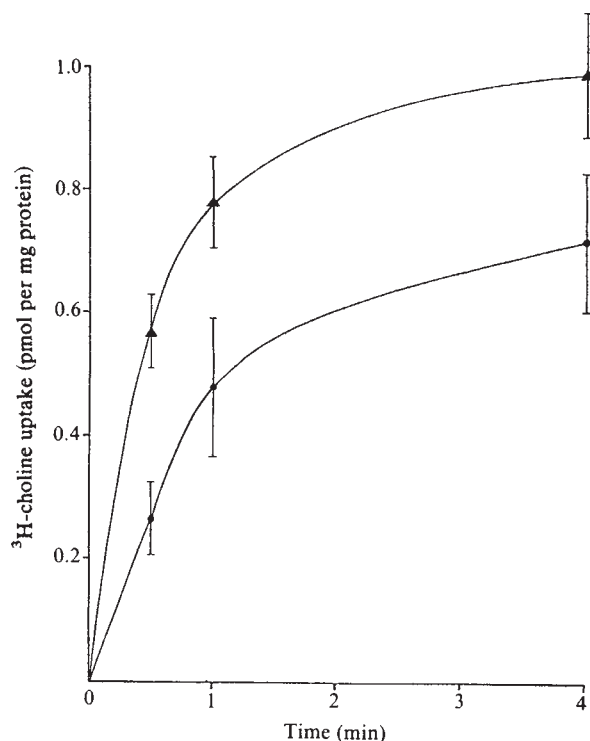


Fig. 1 Time course of uptake of ^3H -choline by liposomes containing membrane extract. Liposomes prepared as described in the text were preincubated for 30 min at 37°C , and then 140- μl samples were added to 70 μl of dialysis fluid (37°C) containing 1–2 μCi ^3H -choline (1 μM). After the required time of incubation (37°C), 200 μl was placed in 5 ml of ice-cold filtering solution (100 mM NaCl, 10 mM Tris-HCl and 10 mM choline chloride, pH 7.4), immediately filtered on Millipore filters (0.22 μm pore diameter) and then washed twice with 10 ml of filtering solution. The order of filtering was randomized because pressure differences in the apparatus affect the blank value. Zero points were obtained by adding the liposome suspension directly to the filtering solution (which already contained ^3H -choline) and filtering immediately. Filters were dried, counted in 10 ml of scintillant and uptake of ^3H -choline determined from the value of the mean of at least four filtrations, minus the zero value. Protein entrapped on the filters was measured directly¹³. ▲, Without 10 mM choline; ●, with 10 mM choline. Vertical bars show s.e.m. of four filtrations.

The effects of varying concentrations of external choline chloride on the uptake of ^3H -choline were investigated and it was found that the uptake was saturable, with a half-maximal concentration of 50–100 μM . Double-reciprocal plots of influx against choline concentration showed an inflection at a choline concentration of about 4 μM . Although we cannot explain this phenomenon, it has also been reported¹¹ in isolated synaptosomes.

In cerebral cortex synaptosomes the initial rate of choline influx at a choline concentration of 1 μM is 10–20 pmol per min per mg protein; the rate in protein-containing liposomes is 0.5–1.0 pmol per min per mg protein, so that only 5–10% of the native activity is retained. This may be because only a fraction of the total carrier has been extracted; alternatively, some may be

denatured, some may be incorporated into the membrane in the wrong orientation or, as occurs with erythrocyte glucose carrier¹², only a small proportion of the liposomes contain a functional carrier molecule. The latter proposal would also explain why inhibition of ^3H -choline influx by 10 mM choline is only seen during the initial phases of uptake. Whatever the explanation, the solubilized carrier when incorporated into liposomes has retained the important properties of saturability, hemicholinium sensitivity and trans-activation.

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1. Sung, C. P. & Johnstone, R. M. *Can J. Biochem.* **43**, 1111–1118 (1965).
2. Hodgkin, A. L. & Martin, K. J. *J. Physiol., Lond.* **179**, 26–27 (1965).
3. Martin, K. J. *gen. Physiol.* **51**, 497–516 (1968).
4. Marchbanks, R. M. *Biochem. J.* **110**, 533–541 (1968).
5. Marchbanks, R. M. & Wonnacott, S. *Prog. Brain Res.* **49**, 77–88 (1979).
6. Jones, D. H. & Matus, A. I. *Biochim. biophys. Acta* **356**, 276–287 (1974).
7. Bardin, C. & Johnstone, R. M., *J. biol. Chem.* **253**, 1725–1732 (1978).
8. Kagawa, Y. & Racker, E. *J. biol. Chem.* **246**, 5477–5487 (1971).
9. Breckenridge, W. C., Gombos, G. & Morgan, I. G. *Biochim. biophys. Acta* **266**, 695–707 (1972).
10. MacIntosh, F. C. *Fedn Proc.* **20**, 562–568 (1961).
11. Yamamura, H. I. & Snyder, S. H. *J. Neurochem.* **21**, 1355–1374 (1973).
12. Goldin, S. M. & Rhoden, V. J. *J. biol. Chem.* **253**, 2575–2583 (1978).
13. Schaffner, W. & Weissmann, C. *Analyt. Biochem.* **56**, 502–514 (1973).

A ribosomal protein mediates eIF-2 phosphorylation by interferon-induced kinase

Kenzo Ohtsuki, Masataka Nakamura, Tsuneaki Koike, Nakao Ishida & Samuel Baron*

Department of Bacteriology, Tohoku University School of Medicine, Sendai 980, Japan

*Department of Microbiology, University of Texas Medical Branch Galveston, Texas 77550

We have previously reported that treatment of mouse L cells with interferon (IFN) induces a reduction of initiation factor (eIF-2) activity (ternary complex formation of eIF-2 with Met-tRNA_i and GTP)¹, and that the reduction of eIF-2 activity involves the phosphorylation of eIF-2 by an IFN-induced protein kinase². In addition, we considered that the reduction of eIF-2 activity induced by the kinase (cyclic AMP-independent protein kinase) may be accompanied by the phosphorylation of some intermediary factor(s)². This possibility was supported by early reports that at least two ribosomal proteins (of molecular weights (MWs) 35,000–38,000 and 64,000–67,000) of mouse L cells^{3–5} and cellular proteins of Ehrlich ascites tumour cells⁶ are highly phosphorylated by IFN treatment. Recently, we and others have purified IFN-induced protein kinases from different cells treated with IFN^{2,3,7–9}. These purified kinases show several similarities. The reports suggested that the IFN-induced kinase may be responsible for the phosphorylation of the ribosomal proteins (MWs 35,000–38,000 and 65,000–67,000) and that the phosphorylation of these proteins by the kinase may be associated with the reduced eIF-2 activity in IFN-treated cells^{2–4,8,9}. A similar inhibitory mechanism of eIF-2 activity has been demonstrated in rabbit reticulocyte lysates^{10–15}. The present study was undertaken to purify a ribosomal protein [MW 65,000 (65K)] from mouse L cells and to examine the effect of the protein on eIF-2 activity, and also on the binding of the ternary complex to the 40S ribosomal subunit. We found that the eIF-2 activity was greatly reduced when both eIF-2 and the ribosomal protein were incubated with IFN-induced kinase *in vitro*. Available evidence suggests that the inhibitory mechanism of eIF-2 activity induced by the kinase *in vitro* may involve phosphorylation of the ribosomal proteins [a small subunit (MW 38,000) of eIF-2 and 65K ribosomal protein] by the IFN-induced kinase.

A 65K ribosomal protein was purified from 0.5 M KCl ribosomal extracts of mouse L cells as described in Fig. 1. SDS-polyacrylamide gel electrophoresis showed that the purified 65K ribosomal protein was a single polypeptide. Table 1 shows that the 65K ribosomal protein functioned as a phosphate acceptor of IFN-induced kinase *in vitro*; however, the substrate activity of the protein was more than 50% of that of purified eIF

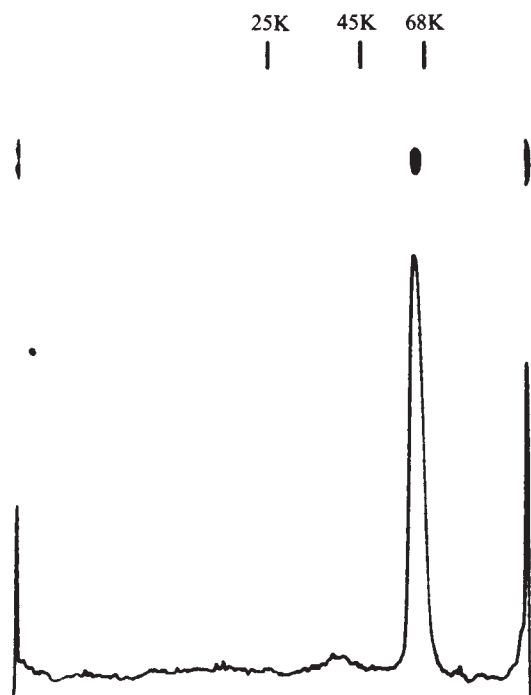


Fig. 1 Purification of the 65K ribosomal protein from 0.5 M KCl extracts of mouse L cells and SDS-polyacrylamide gel electrophoresis of the protein. 0.5 M KCl extracts from ribosomes of mouse L cells were used for the purification of 65K ribosomal protein because early reports showed that at least two proteins (MWs 35,000–38,000 and 64,000–67,000) are highly phosphorylated in the cell extracts of IFN-treated cells and this protein phosphorylation is greatly stimulated by the addition of dsRNA (poly(I)·poly(C), 10–200 ng ml⁻¹)^{2,3,6}. The 0.5 M KCl extract (about 300 mg crude protein) was passed through a Sephacryl S-200 column (2.2 × 76 cm) previously equilibrated with buffer A [20 mM Tris-HCl (pH 7.5) containing 2 mM dithiothreitol, 50 mM KCl and 5% glycerol] to purify a 65K ribosomal protein which was highly phosphorylated by IFN-induced kinase *in vitro*. The column was eluted with buffer A and 1.0 ml of fraction was collected. The fractions (MWs ~60,000–70,000) were pooled and concentrated with solid ammonium sulphate (60% saturation). After dialysis against buffer A (overnight at 4°C), the fraction (~60 mg protein) was further purified by a DEAE-cellulose column (1.4 × 12 cm) previously equilibrated with buffer A. The column was eluted with a linear gradient from 0.05 M to 0.5 M KCl in buffer A (50 ml). DEAE-cellulose column chromatography showed two distinct protein peaks (flow-through fraction and the 0.08 M–0.35 M KCl fraction). Over 70% protein kinase activity of the Sephacryl S-200 fraction was detected in the flow-through fraction, whereas the stimulating effect of dsRNA (poly(I)·poly(C), 100 ng ml⁻¹) on substrate activity for the kinase was detected in the fractions which were eluted between 0.1 M and 0.15 M KCl. Moreover, the activity of cyclic AMP-independent protein kinase was eluted between 0.15 M and 0.2 M KCl. The fractions with substrate activity for the kinase were pooled and immediately concentrated by vacuum dialysis against buffer A to 0.5 ml. Finally, the DEAE-cellulose fraction (total 15 mg protein) was purified by preparative gel electrophoresis. The protein with substrate activity for the kinase in the gel was extracted with 0.5 M KCl extracts from the ribosomes of mouse L cells with a protein yield of less than 1%. SDS-polyacrylamide gel electrophoresis¹⁶ showed that the purified ribosomal protein was a single polypeptide with a MW of 65,000, as estimated from the marker proteins.

(98% pure). About 80 pmol of the ribosomal protein were phosphorylated in these conditions. In addition, double-stranded (ds) RNA (poly(I)·poly(C), 100 ng ml⁻¹) stimulated about 3.2 times the phosphorylation of the ribosomal protein by the kinase *in vitro*. The phosphorylation of the 65K ribosomal protein and eIF-2 was confirmed by SDS-polyacrylamide gel electrophoresis (data not shown). No protein kinase activity in the final preparation of 65K ribosomal protein was detected.

We tested the effect of 65K ribosomal protein on eIF-2 activity. Less than a 9% reduction was detected when purified eIF-2 (98% pure) was incubated with the kinase in the absence of the ribosomal protein, whereas the activity was greatly reduced when both eIF-2 and the ribosomal protein were preincubated with the kinase. No reduction of eIF-2 activity occurred when both proteins were incubated without the kinase (Table 2). In addition, no reduction of eIF-2 activity occurred when ATP or Mg²⁺ was omitted from the reaction mixture. The effect of ATP was not substituted by GTP and high levels of Mg²⁺ (>5 mM) prevented the reduction of eIF-2 activity induced by the phosphorylation of these two proteins (eIF-2 and 65K ribosomal protein) by the kinase *in vitro* (data not shown). Low levels of dsRNA stimulated this reduction, but it was prevented by aminopurine which is an inhibitor of the kinase^{2,17}. These results showed that the phosphorylation of these two ribosomal proteins by the kinase *in vitro* resulted in the reduction of eIF-2 activity.

Table 1 Effect of dsRNA on phosphorylation of eIF-2 or 65K ribosomal protein by IFN-induced kinase *in vitro*

Additions	[γ- ³² P] ATP into the substrate (pmol)
None	0.1
eIF-2	15.0
eIF-2 + dsRNA	31.3
65K ribosomal protein	8.9
65K ribosomal protein + dsRNA	28.5
65K ribosomal protein + eIF-2	22.6
65K ribosomal protein + eIF-2 + dsRNA	54.0
65K ribosomal protein + aminopurine	3.8

The reaction mixture (0.1 ml) contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 1 mM magnesium acetate, 0.1 mM [γ-³²P] ATP (3,000 c.p.m. pmol⁻¹), 10 μg of bovine serum albumin (BSA), 0.5 μg of IFN-induced kinase and a substrate [5 μg of purified eIF-2 (98% pure) or 5 μg of 65K ribosomal protein]. The reaction mixture was incubated for 10 min at 37°C in the presence or absence of dsRNA (poly(I)·poly(C), 100 ng ml⁻¹) or 1 mM aminopurine. The reaction was stopped by the addition of 0.5 ml of 20% trichloroacetic acid (TCA) and 0.5 ml of 0.2 M sodium pyrophosphate containing 1 mg ml⁻¹ of BSA. The radioactivity of the TCA precipitate was determined as previously reported⁸.

In the presence of GTP and Met-tRNA_i, eIF-2 forms the ternary complex, Met-tRNA_i-eIF-2-GTP, which binds to the 40S ribosomal subunit^{13,15}. We tested the effect of 65K ribosomal protein on the binding of the ternary complex to the 40S subunit. The complex (ternary complex-40S subunit) formed was analysed by 5–30% (w/v) sucrose density gradient centrifugation. Figure 2 shows that the complex formation was greatly reduced when both eIF-2 and 65K ribosomal protein were preincubated with the kinase in the presence of ATP (0.1 mM) and Mg²⁺ (1 mM). The reduction of the complex formation did not occur if ATP or Mg²⁺ was omitted from the reaction mixture, as described in the reduction of eIF-2 activity induced by the kinase. These results suggested that the phosphorylation of two ribosomal proteins by the kinase results in the reduction of the binding of the ternary complex to the 40S subunit.

The present finding that the reduction of eIF-2 activity involves phosphorylation of both eIF-2 and 65K ribosomal protein by IFN-induced kinase *in vitro* is at least partially

consistent with other observations which are: (1) at least two ribosomal proteins (MWs 35,000–38,000 and 64,000–67,000) which are highly phosphorylated by IFN treatment^{3–5}; (2) low levels of dsRNA stimulated the phosphorylation of a small subunit (MW 38,000) of eIF-2 and 65K ribosomal protein by the kinase *in vitro*^{2,4,17,18}; and (3) in rabbit reticulocyte lysates, the eIF-2 activity is inhibited by a haem regulatory inhibitor (HRI, cyclic AMP-independent protein kinase)^{10–12}. However, it has also been reported that no inhibition occurs when highly purified eIF-2 is used^{19,20}. In these conditions, HRI phosphorylates only a small subunit of eIF-2, thereby indicating that the phosphorylation of a small subunit of eIF-2 alone is not sufficient to alter the ternary complex forming activity of eIF-2^{19,20}. Our data and indirect evidence in the literature on reticulocyte lysates strongly suggest that the 65K ribosomal protein may be the intermediary factor which is responsible for the reduction of eIF-2 activity induced by IFN treatment or

Table 2 Effect of 65K ribosomal protein on eIF-2 activity

Preincubation	[³⁵ S] Met-tRNA into ternary complex (pmol)
Without kinase	
Complete (control I)	23.7
Complete + 65K ribosomal protein (1 µg)	22.5
Complete + 65K ribosomal protein (5 µg)	21.3
With kinase	
Complete (control II)	21.8
Complete + 65K ribosomal protein (5 µg)	12.0
Complete + 65K ribosomal protein (5 µg) + dsRNA (100 ng ml ⁻¹)	6.3
Complete + 65K ribosomal protein (5 µg) + amino-purine (2 mM)	16.7
Complete + dsRNA (100 ng ml ⁻¹)	19.7
Complete + aminopurine (2 mM)	20.0

The complete reaction mixture (0.1 ml) contained 20 mM Tris-HCl (pH 7.5), 4 mM dithiothreitol, 1 mM MgCl₂, 10 µg BSA, 0.1 mM ATP and 5 µg (bound with 23.7 pmol of [³⁵S]Met-tRNA_f) of purified eIF-2 (98% pure). The indicated concentrations of the reagent and eIF-2 (5 µg) were incubated for 10 min at 37 °C with 65K ribosomal protein (5 µg) in the presence or absence of IFN-induced kinase (0.5 µg). After incubation, the mixture containing 50 pmol of [³⁵S]Met-tRNA_f (4,500 c.p.m. pmol⁻¹), 0.1 M KCl and 0.1 mM GTP was added to the reaction mixture and then further incubated for 10 min at 0–5 °C. The formed ternary complex of eIF-2 was determined as previously reported². The eIF-2 from ribosomes of mouse L cells and IFN-induced kinase from ribosomes of mouse L cells treated with IFN (500 units for 24 h at 37 °C) were prepared as previously reported².

IFN-induced kinase *in vitro*. In addition, the recent evidence of a steric difference of a 5'-terminal structure between viral and eukaryotic cell mRNAs suggests that the phosphorylation of ribosomal proteins (eIF-2, 65K ribosomal protein and others) by IFN-induced kinase may be responsible for the discrimination of initiation complex formation between viral and eukaryotic cell mRNAs²¹. We are now carrying out comparative and analytical studies on the biological and physicochemical properties of the 65K ribosomal protein from mouse L cells treated with IFN and the cells infected with viruses.

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- Ohtsuki, K., Dianzani, F. & Baron, S. *Nature* **269**, 536–538 (1977).
- Ohtsuki, K. & Baron, S. *J. Biochem. Tokyo* **85**, 1495–1502 (1979).
- Zilberstein, A., Federman, R., Shulman, L. & Revel, M. *FEBS Lett.* **68**, 119–124 (1976).
- Zilberstein, A., Kimchi, A., Schmidt, A. & Revel, M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4734–4738 (1978).
- Gupta, S. *J. Virol.* **29**, 301–311 (1979).
- Lebleu, B., Sen, G. C., Shaila, S., Cabrera, B. & Lengyel, P. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3107–3111 (1976).
- Sen, G. C., Taira, H. & Lengyel, P. *J. biol. Chem.* **253**, 5915–5921 (1978).
- Baglioni, C. *Cell* **17**, 255–264 (1979).
- Kimchi, A., Zilberstein, A., Schmidt, A., Shulman, L. & Revel, M. *J. biol. Chem.* **254**, 9846–9853 (1979).
- Datt, A., Haro, C. D., Sierra, J. M. & Ochoa, S. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1463–1467 (1977).
- Datt, A., Haro, C. D. & Ochoa, S. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1148–1152 (1978).
- Lenz, J. R. & Baglioni, C. *J. biol. Chem.* **253**, 4219–4223 (1978).
- Petryshyn, R., Trachsel, H. & London, I. M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1575–1579 (1979).
- Ernst, V., Levin, D. H. & London, I. M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2118–2122 (1979).
- Haro, C. D. & Ochoa, S. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1741–1745 (1979).
- Weber, K. & Osborn, M. *J. biol. Chem.* **244**, 4406–4412 (1969).
- Farrell, P. J., Balkow, K., Hunt, T. & Jackson, R. *Cell* **11**, 187–200 (1977).
- Ohtsuki, K. *J. clin. Oncol. Hemat.* **10** (in the press).
- Tahara, S. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 789–793 (1978).
- Trachsel, H. & Staehelin, T. *Proc. natn. Acad. Sci. U.S.A.* **75**, 204–208 (1978).
- Kozak, M. *Cell* **15**, 1109–1123 (1978).

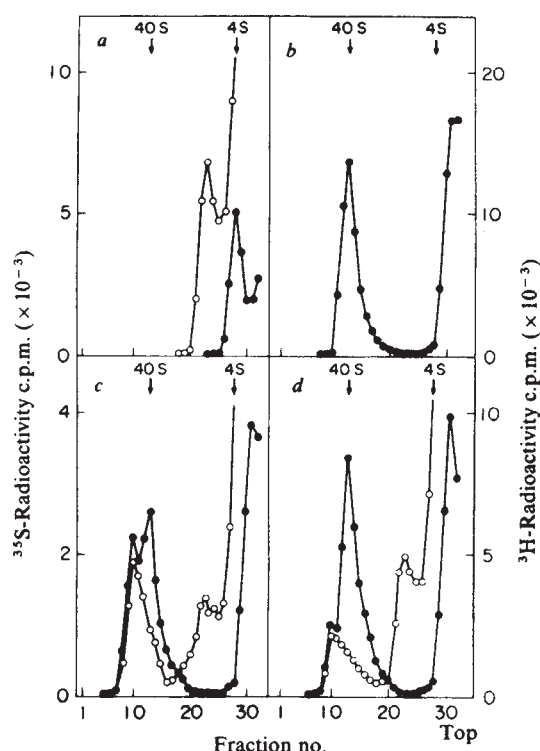


Fig. 2 Effect of 65K ribosomal protein on the binding of the ternary complex to the 40S ribosomal subunit. The reaction mixture (0.1 ml) contained 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, 0.1 mM GTP, 50 pmol of [³⁵S]Met-tRNA_f (4,500 c.p.m. pmol⁻¹), 10 pmol of [³H] 40S subunit (0.16 A₂₆₀ units), 1 mM MgCl₂ and purified eIF-2 (5 µg). Incubation was for 10 min at 37 °C. The reaction mixtures were analysed 5–30% (w/v) sucrose density gradient (4.8 ml) in 20 mM Tris-HCl (pH 7.5), 0.1 M KCl and 1 mM MgCl₂. Centrifugation was at 45,000 r.p.m. in a Hitachi 50 SW rotor for 3 h at 4 °C. The gradients were divided separately into 10-drop fractions from the bottom of the tube. Radioactivities of ³H and ³⁵S of the fractions were determined in a liquid scintillation spectrometer. *a*, ³⁵S radioactivity of the formed ternary complex [³⁵S]-Met-tRNA_f-eIF-2-GTP, and ³H-Met-tRNA_f (1,300 c.p.m. pmol⁻¹, 30 pmol) after gel filtration of Sephadex G-25 to remove free ³H-methionine; *b*, ³H radioactivity of ³H-40S subunit; *c*, the binding of the ternary complex to the ³H-40S subunit (10 pmol). A ternary complex was formed in the condition as previously reported⁴. The binding assay was carried out in the presence of 65K ribosomal protein (5 µg) without IFN-induced kinase. *d*, Purified eIF-2 (98% pure, 5 µg) and 65K ribosomal protein (5 µg) were preincubated with the kinase (0.5 µg) in the presence of ATP (0.1 mM). After incubation (10 min at 37 °C), the reaction mixture was added to the binding assay mixture and then incubated for 5 min at 37 °C. ●, ³H; ○, ³⁵S.

An interferon-induced cellular enzyme is incorporated into virions

David Wallach & Michel Revel

Department of Virology, The Weizmann Institute of Science, Rehovot, Israel

The mechanisms by which interferon inhibits viral growth are only partially understood. Several enzymatic activities increase in cells shortly after treatment with interferon¹⁻⁸. One of these enzymes, oligo-isoadenylate synthetase, synthesizes (2'-5') isoadenylate oligomers which strongly stimulate the activity of a cellular ribonuclease, RNase F (ref. 7). Interferon also significantly increases the activity of a protein kinase which phosphorylates the initiation factor eIF-2 and can inhibit *in vitro* protein synthesis. Such interferon-induced enzymes, which affect RNA and protein metabolism, might be responsible for many of its effects on viruses. Indeed, inhibition of viral protein and RNA synthesis appears to have a major role in the antiviral state⁹. We have now investigated possible interactions of the two enzymes with viral constituents during the course of infection and found that in two different membrane-coated RNA viruses, vesicular stomatitis virus (VSV) and Moloney murine leukaemia virus (M-MuLV), there is an accumulation of the (2'-5') oligo-isoadenylate synthetase (E) in the virions. Most of the enzyme is bound to the virion ribonucleoprotein core. The incorporation of E into the virions suggests a direct involvement of the enzyme in regulation of virus functions.

Highly-purified VSV and M-MuLV virions were lysed in NP40, and after mixing with poly(rI):poly(rC)-agarose beads were incubated with [α -³²P]ATP. Figure 1 shows the autoradiography of an electrophoretic analysis of the labelled nucleotides formed with VSV virions, grown in BSC-1 monkey fibroblasts and with M-MuLV virions, from chronically infected NIH/3T3 mouse fibroblasts. The nucleotides formed during incubation of ATP with the respective cell extracts are shown in comparison. Equal amounts of proteins were used in the assay of the virions and of the cellular extracts. Before electrophoresis the labelled nucleotides were treated with bacterial alkaline phosphatase so that we analysed the phosphatase-resistant cores of the nucleotides formed by E comprising (2'-5')ApA; (2'-5')A(pA)₂ and so on.

Figure 1 (lanes 3, 4) shows that purified VSV virions, formed in BSC-1 cells, exhibit a high activity of E. The activity in the virions was proportional to that in the cells. BSC-1 cells which have not been treated with interferon showed low activity of E (Fig. 1, lane 1). Extracts of virions obtained from these untreated cell cultures also formed very little oligo-isoadenylate, but on a protein basis, the activity of E in the virions was about ninefold higher than in the cells (Fig. 1, lane 3). Treatment by 15 U per ml of interferon increased the activity of the enzyme in the cells and in the virions by about 10-fold (Fig. 1, lanes 2, 4). M-MuLV virions, and their host cells (NIH/3T3) showed no activity of E before treatment with interferon (Fig. 1, lanes 5, 7). Following treatment with interferon there was high activity of E in both the cells and the virions (Fig. 1, lanes 6, 8). The length of the oligo-isoadenylate formed by E is proportional to the activity of the enzyme. Thus, only the shortest oligomer, (2'-5')ApA, is formed in the presence of low concentrations of lysed VSV virions (Fig. 1), while in the presence of higher concentrations of virion lysates there is also formation of (2'-5')ApApA (Fig. 2b, lane 2). Further characterization of the nucleotides formed by VSV virions, is shown in Fig. 2. The products not treated with phosphatase were fractionated on a DEAE-cellulose column, in urea, from which, as shown before⁷, dimer and trimer oligo-isoadenylates (2'-5')pppApA and pppApApA elute sequentially as distinct peaks after the ATP

Table 1 Fractionation of NP40-treated VSV virions

	E activity		Protein composition (% of total protein in the fraction)			
	(c.p.m. in (2'-5')ApA)					
	No NP40	+NP40	L	G	N+NS	M
Intact virions	200	2400	2	22	39	35
NP-40 extract	—	230	<1	36	<1	57
NP-40 insoluble cores	1600	1600	3	6	67	25

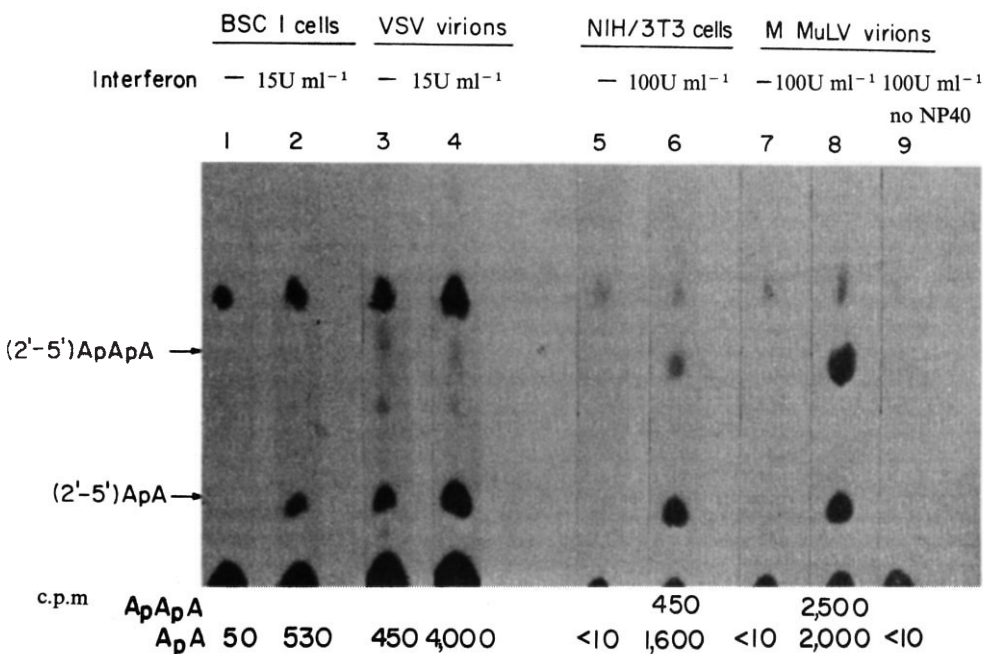
Purified VSV virions (200 μ g protein), labelled *in vivo* by ³H-uridine, were suspended in 100 μ l of 0.2% (v/v) NP40, 120 mM KCl, 5 mM MgCl₂, 7.5 mM β -mercaptoethanol and 10 mM HEPES buffer, pH 7.5. After incubation for 20 min at 4 °C they were applied on a 4-ml cushion of the same solution, but without NP40 and with 20% (w/v) sucrose, and spun for 60 min at 200,000 g. The proteins which did not sediment through the cushion were collected and the pelleted ribonucleoprotein cores were resuspended to the original volume of the virion sample in the above solution. Samples (5 μ l) of the virions, of virion without NP40, of the non-sedimented proteins and of the NP40-insoluble proteins were assayed for E in the presence of poly(rI):poly(rC)-agarose as described in Fig. 1. The patterns of proteins in the virions and in the two fractions of the detergent-treated virus were analysed by SDS-electrophoresis on 7-18% gradient polyacrylamide gel and quantitated by densitometry. Quantification of the uridine label confirmed that at least 90% of the viral RNA was recovered in the NP40-insoluble pellet.

(Fig. 2a, peaks I, II). In high voltage paper electrophoresis at pH 3.5 the nucleotides of peaks I and II migrated ahead of ADP, as the authentic oligo-isoadenylates^{4,7} (not shown). Following treatment by phosphatase, the phosphatase-resistant cores co-migrated in the paper electrophoresis with (2'-5') oligo A dimer and trimer markers and were clearly distinguished from (3'-5') oligo A markers and from 5'-AMP (Fig. 2b, lanes 3, 4).

Although we used no direct visualization technique to confirm the localization of E in the virions, the following arguments eliminate the possibility that the virion activity results from contamination by cellular proteins. As shown in Fig. 1, lane 9, the M-MuLV virions showed very little activity of E unless they were disrupted by detergent. The activity of VSV showed a similar dependence on detergent treatment (Table 1). The enzyme is, therefore, found inside the virion and not just adsorbed on its surface. It is also very unlikely that the enzyme is found inside some cellular sub-particle which would co-purify with the virions, since the density at which VSV virions band is significantly different from the density of M-MuLV. Finally, as shown in Fig. 3, the activity of E fully coincides with the virion peak on sucrose gradient. Three successive sedimentation bandings of purified VSV virions in 20-30% sucrose gradients (between each the virions were dispersed by sonication) did not change the specific activity of E in the virions.

To examine the nature of the association between E and the virus we disrupted VSV virions by NP40 and separated the solubilized proteins from the RNA and its associated proteins (Table 1). The NP40-soluble fraction contained only the membrane-associated G protein and the M protein. Only little E activity was found in this fraction. The majority of the activity of E, originally found in the virions, was recovered in the detergent-resistant fraction (Table 1). This activity, in contrast to the activity of E in the intact virions, was not dependent on the addition of detergent. The detergent-resistant fraction contained all of the RNA-associated proteins L, N and NS, but significantly decreased amounts of the M proteins and very little G protein. Further fractionation of the NP40-insoluble core is necessary to identify the site at which E is bound; E has been shown to bind to double-stranded RNA and its activity is strongly stimulated by double-stranded RNA^{6,10}. It seems unlikely that, in the virion, the enzyme is bound to a double-stranded portion of the RNA since the enzyme present in the virion was not active without the addition of the synthetic double-stranded RNA poly(rI):poly(rC). Full activation occurred at concentrations of 1-10 μ g ml⁻¹ poly(rI):poly(rC) (not shown). Preliminary results suggest that there is a difference between the affinity of virion-associated E and of cellular E for double-stranded RNA. At 0.12 M KCl, E from VSV virion

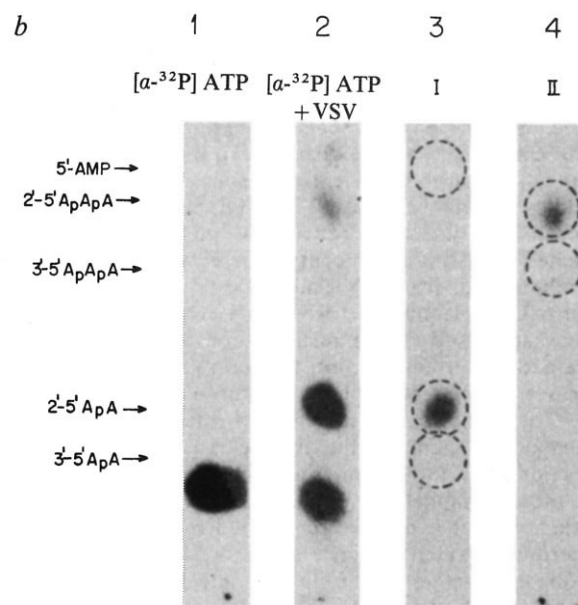
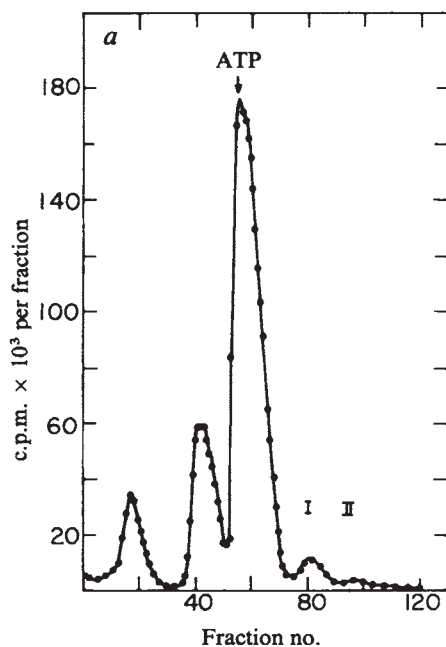
Fig. 1 Electrophoretic pattern of ATP-derived nucleotides formed by disrupted virions and by extracts of the infected cells. VSV (Indiana type, thrice plaque-purified) was collected 12 h after infecting BSC-1 cells with 0.02 plaque forming units (PFU) per cell. Following sedimentation of cell debris at low speed, the virus was sedimented through a cushion of 20% (w/v) sucrose in Dulbecco's phosphate-buffered saline (PBS) and then purified by velocity sedimentation banding in a 20–35% (w/v) sucrose gradient in PBS plus 2 mM EDTA followed by banding in a 20–40% (w/v) tartrate, 20%–0% (w/w) glycerol gradient in 20 mM Tris; 0.5 mM EDTA pH 7.4. Following each step the virus was dispersed by brief sonication. The virions formed a single sharp band on the gradients. M-MuLV, released by chronically infected NIH/3T3 cells¹⁸ during a 24-h period, was purified by passing the cell-growth medium through a 0.45 μ m Millipore filter, sedimenting the virus on a cushion of 60% sucrose and then banding in a 20–60% (w/v) sucrose gradient which also contained 10 mM Tris-HCl pH 7.4, 1 mM EDTA and 100 mM NaCl. Interferon was added to BSC-1 cells 24 h before the infection (15 U ml^{-1} human leukocyte interferon induced by Sendai virus in Namalva lymphoblastoid cells). NIH/3T3 cells were treated with 100 U ml^{-1} mouse interferon (induced by NDV in L929 fibroblasts) and 12 h later the medium was changed and interferon was re-added. Virions were collected 24 h after medium change. Both interferons were purified to about $10^7 \text{ U per mg protein}$. The purified virions were incubated for 20 h at 30°C , with poly(rI):poly(rC)-agarose beads (PL Biochemicals) in the presence of 0.1% NP40 (except for lane 9), 1.25 mM [α - ^{32}P]ATP (100 – $300 \text{ mCi mmol}^{-1}$), 5 mM magnesium acetate, 120 mM KCl, 1 mM dithiothreitol (DTT), 20 mM HEPES buffer, pH 7.5 and 10% glycerol. Following incubation, samples of the labelled products were treated by bacterial alkaline phosphatase and analysed by high-voltage paper electrophoresis at pH 3.5 using (2'-5')ApA and (2'-5')ApApA markers. E activity in cells was determined in the same conditions using NP40 extracts of cells prepared and pre-adsorbed on poly(rI):poly(rC)-agarose beads as described previously⁶. Equal amounts of viral and cellular proteins were applied on the beads ($3 \mu\text{g}$ VSV and BSC-1 monkey cell proteins and $10 \mu\text{g}$ of M-MuLV and of chronically infected NIH/3T3 cell proteins). The labelled spots revealed in the autoradiogram given here, were cut out and counted with toluene scintillation fluid. The counts in the (2'-5')ApA and (2'-5')ApApA spots are given at the bottom of the figure. A background of 30 c.p.m. was subtracted.



lysates was readily eluted from poly(rI):poly(rC)-agarose beads. In the same conditions the enzyme of the BSC-1 cell extract remained adsorbed to the double-stranded RNA (not shown). The reason for the different behaviour of the virion-associated enzyme is not clear; it raises however the possibility

that the interaction of E with viral components interferes with its binding to poly(rI):poly(rC). The mere fact that E is efficiently incorporated into the virions is also suggestive of some association between the enzyme and viral components. Without such an association it is difficult to see how E can be incorporated into

Fig. 2 Characterization of the oligo-isoadenylate synthesized by the disrupted virions. *a*, Chromatography on DEAE-Sephacel, of the nucleotides formed in incubation of lysed virions with [α - ^{32}P]ATP. *b*, Electrophoretic analysis, following phosphatase treatment, of the nucleotides formed during the incubation with the virions (lane 2) and of fractions I and II (lanes 3, 4) from the DEAE-Sephacel chromatography. Unlabelled nucleotides, detected by UV light, are indicated by dotted lines [5'-AMP (2'-5')ApA and (3'-5')ApA in lane 3 and (2'-5')ApApA and (3'-5')ApApA in lane 4]. Lane 1 shows that the slowest migration spot is a contaminant of the [α - ^{32}P]ATP preparation. A sample of purified VSV virions ($80 \mu\text{g protein}$) was incubated with: $100 \mu\text{g ml}^{-1}$ poly(rI):poly(rC); 1.25 mM [α - ^{32}P]ATP, 5 mM MgCl_2 ; 7 mM DDT, 20 mM HEPES, pH 7.5; 50 mM KCl; 10% glycerol and 0.1% NP40 in a final volume of $40 \mu\text{l}$ for 24 h at 30°C . The nucleotides were then applied on a $0.7 \times 16 \text{ cm}$ DEAE-Sephacel column and eluted by a 50–250 mM NaCl gradient in 7 M urea and 50 mM Tris-HCl, pH 7.5 buffer. Fractions of 0.5 ml were collected and counted, without scintillation, in the ^3H -channel of a scintillation counter (*a*). The fractions in peak I and II were pooled, de-salted and concentrated by adsorption on a small DEAE-Sephacel column and then eluted by 1 M NH_4HCO_3 . The nucleotides were treated by bacterial alkaline phosphatase and analysed by high-voltage paper electrophoresis as before⁴ (*b*).



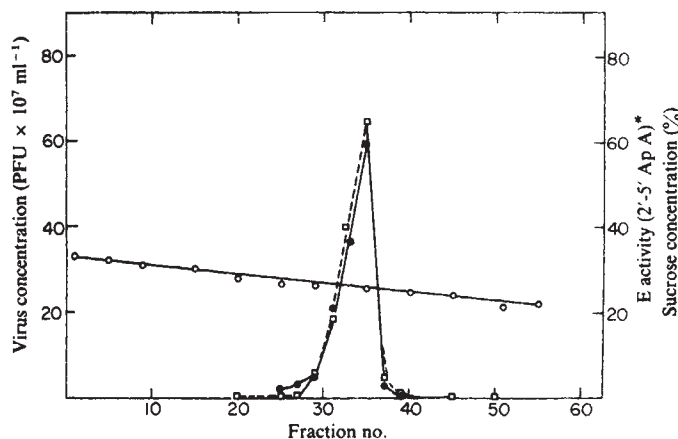


Fig. 3 Co-sedimentation of VSV and E activity. ●, Virus concentration; □, E activity; ○, sucrose concentration. A sample of purified VSV virions (100 µg protein) was applied on a sucrose gradient (13 ml 35%–20% w/v in PBS and 2 mM EDTA) and spun for 1 h at 100,000 g. Fractions of 0.3 ml were collected and the amount of virus in each fraction was determined by plaque assay on L929 fibroblasts. Sucrose concentration in the fractions was determined by refractometry. A sample of each fraction was diluted in PBS and spun again for 2 h at 250,000g. The pelleted virus was then resuspended in 40 µl of a solution of 120 mM KCl, 5 mM magnesium acetate, 1 mM DTT, 10% glycerol and 20 mM HEPES buffer, pH 7.5 and incubated for 18 h at 30 °C with 2.5 mM [α -³²P]ATP, 100 µg ml⁻¹ poly(rI):poly(rC) and 0.1% NP40. Following phosphatase treatment, a sample was analysed by high-voltage electrophoresis followed by an autoradiography and spectrophotometric scanning of the (2'-5')ApA spots. E activity is expressed as the weight (in mg) of the paper under each scanning peak.

the virions to give activity which exceeds that in the cell, while most other cellular proteins are efficiently excluded from the virions in their assembly.

An interesting implication of the fact that E binds to viral components is that in virus-infected, interferon-treated cells, E is not likely to be evenly distributed but rather to be accumulated in subcellular sites in which there is assembly and synthesis of those viral components to which E can bind. Such uneven distribution of E in the cell might be an important factor in the function of this enzyme, and may result in a stronger effect of E on viral functions. It can also limit the effects of E on the cellular functions since degradation of the oligo-isoadenylate by the 2'-phosphodiesterase⁷ might limit their effects [that is, activation of RNase F (ref. 7)] to the site of their synthesis in the cell. As has been pointed out by others, the strong stimulation of E activity by double-stranded RNA, formed during RNA replication of many viruses, might be an important factor in the specificity of the oligo-isoadenylate dependent ribonuclease¹¹. Association between cellular and viral components, as part of the interferon-induced antiviral state, was also suggested in studies on other viruses. Thus, Galster and Lengyel¹² have shown that uncoated reoviruses, purified from interferon-treated (but not from control) infected cells are unable to synthesize full-size mRNA molecules in spite of their apparently normal double-stranded RNA and viral protein composition. They suggested that a nuclease, or a factor which induces premature termination of transcription associates with the viruses upon infection of the interferon-treated cells. Aujean *et al.*¹³ have shown that infection of interferon-treated cells by Mengo virus increased the ribonuclease activity in a subcellular fraction known to contain the viral replication complex.

Another implication of the fact that E accumulates in the virions is that E can be transported by the virion itself from interferon-treated to untreated cells. Inside the virion, E is probably inactive but, following infection and uncoating of virions which contain E, the enzyme might synthesize oligo-isoadenylate in close proximity to the viral RNA. Since cells, including those which have not been treated by interferon, contain RNase F which is activated by very low concentrations of oligo-isoadenylate⁷, uncoating of the virus which contains E may eventually lead to its own destruction. Virions of MuLVs,

formed by interferon-treated cells, have indeed been shown to have reduced infectivity^{14,15}. It is possible that the defectiveness of these virions is not due to the alteration of a viral product^{16,17}, but rather to the incorporation into the virion of an interferon-induced cellular protein which interferes with the normal function of the virus.

So far, the study of the proteins induced by interferon concerned mainly their enzymatic activities. The presence of high amounts of E in virions suggests that besides their activity, the subcellular localization of these proteins and the mode of their interaction with the viral components can be of major importance in determining the way by which they protect cells against viruses.

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1. Roberts, W. K., Hovanessian, A., Brown, R. E., Clemens, M. J. & Kerr, I. M. *Nature* **264**, 477–480 (1976).
2. Revel, M. *et al.* in *Proc. 11th FEBS Meet.* **43**, 47–58 (1977).
3. Kerr, I. M. & Brown, R. E. *Proc. natn. Acad. Sci. U.S.A.* **75**, 256–260 (1978).
4. Zilberstein, A., Kimchi, A., Schmidt, A. & Revel, M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4734–4738 (1978).
5. Farrell, P. J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **75**, 5893–5897 (1978).
6. Kimchi, A. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **76**, 3208–3212 (1979).
7. Schmidt, A. *et al.* *FEBS Lett.* **95**, 257–264 (1978).
8. Cooper, J. A. & Farrell, P. J. *Biochem. biophys. Res. Commun.* **77**, 124–131 (1977).
9. Friedman, R. M. & Chang, E. H. in *Interferons and their Actions* (ed. Stewart, W. E. II) 145–152 (CRC Press, Ohio, 1977).
10. Hovanessian, A. G., Brown, R. E. & Kerr, I. M. *Nature* **268**, 537–539 (1977).
11. Nilsen, T. W. & Baglioni, C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2600–2604 (1979).
12. Galster, R. L. & Lengyel, P. *Nucleic Acids Res.* **3**, 581–598 (1976).
13. Aujean, O., Sanceau, J., Falcoff, E. & Falcoff, R. *Virology* **92**, 583–586 (1979).
14. Pitha, P. M., Rowe, W. P. & Oxman, M. N. *Virology* **70**, 324–338 (1976).
15. Wong, P. K. Y. *et al.* *Cell* **10**, 245–252 (1977).
16. Chang, E. H. & Friedman, R. M. *Biochem. biophys. Res. Commun.* **77**, 392–398 (1977).
17. Bandyopadhyay, A. K., Chang, E. H., Levy, C. C. & Friedman, R. M. *Biochem. biophys. Res. Commun.* **87**, 983–988 (1979).
18. Salzberg, S., Bakhanashvili, M. & Aboud, M. J. *gen. Virol.* **40**, 121–130 (1978).

Identification of the lethal target of benzylpenicillin in *Streptococcus faecalis* by *in vivo* penicillin binding studies

R. Fontana*, P. Caneparit†, G. Satta† & J. Coyette‡

*Istituto de Microbiologia, Università di Padova, Padova, Italy

†Istituto di Microbiologia, Università di Genova, Genova, Italy

‡Service de Microbiologie, Faculté de Médecine, Université de Liège, Liège, Belgium

The mode of bacterial killing by penicillins is still unknown in spite of many studies on the subject. The recent finding of multiple penicillin binding proteins (PBPs) in sensitive bacteria and the possibility of analysing the binding of the antibiotic to exponentially growing cells have provided new directions for investigating this problem^{1–3}. Sensitivity to lethal and other effects of penicillin varies very significantly with the conditions of growth of the cells. If PBPs were the penicillin target, changes in conditions of growth causing variations in penicillin sensitivity should be accompanied by changes in these proteins. Furthermore, if one of PBPs could be identified as the killing target, it could possibly be demonstrated to show changes in cells growing in different conditions. We show here that in *Streptococcus faecalis* ATCC 9790 changes in conditions of growth are accompanied by changes in PBPs. Furthermore, in the presence of the minimal dose of ¹⁴C-benzylpenicillin causing complete inhibition of cell growth, 100% of the total radioactivity is bound to a single protein (PBP 3).

To study the effect of benzylpenicillin on cells growing in different conditions, we have incubated the cells in the chemically defined medium (CDM) described by Shockman⁴ at the

temperature optimal for growth (45 °C, generation time 31.5 min) and at a temperature allowing a 1.5-fold lower growth rate (32 °C, generation time 47.27 min).

At 32 and 45 °C, the minimal doses of benzylpenicillin which inhibited cell growth (MIC) were 4 and 0.04 $\mu\text{g ml}^{-1}$, respectively, thus confirming the apparent direct dependence of penicillin sensitivity of *S. faecalis* cells on the growth rate.

Membranes from cells growing exponentially at 32 and 45 °C were treated with ^{14}C -benzylpenicillin and PBPs detected by polyacrylamide gel electrophoresis and fluorography as described in Fig. 1 legend. As Fig. 1 and Table 1 show, for most of the proteins, the proportion of the total radioactivity bound by the same protein at the two temperatures is clearly different. Note that the various PBPs respond in different ways to the changes of temperature: PBPs 1, 2 and 3 are present in a larger relative amount in membranes from cells grown at 45 °C than at 32 °C, whereas an increase in the relative amount of PBP 6 is found at the latter compared with the former temperature. This indicates that the changes in PBPs are not due to a nonspecific effect of temperature on these proteins. This was confirmed by the fact that, regardless of growth temperature, binding of

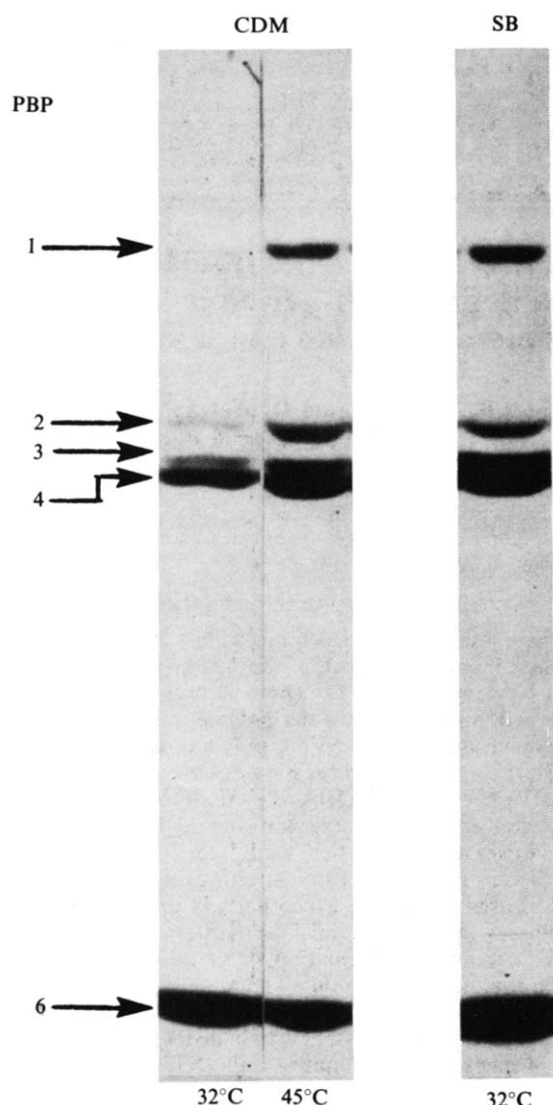


Fig. 1 Penicillin binding proteins of *S. faecalis* grown in different conditions. Membranes were prepared from cells exponentially growing in CDM (chemically defined medium) and in SB (streptococcal broth) and treated with 20 μM ^{14}C -benzylpenicillin (58 Ci mol^{-1}) at 37 °C for 15 min as described in ref. 5. The proteins were then fractionated by SDS-polyacrylamide gel electrophoresis¹³ and the PBPs detected by fluorography¹⁴. PBP 5 has not been detected on these gels; this protein, like the PBPs 7 and 8 of *E. coli*¹², is not consistently observed.

Table 1 Per cent of radioactivity bound to PBPs in *S. faecalis* grown at 32 and 45 °C in CDM

		Per cent of radioactivity bound to				
		PBP 1	PBP 2	PBP 3	PBP 4	PBP 6
Isolated membranes	A	1.5	2.7	3.4	24.9	67.2
	B	8.7	11.4	15.7	26.6	37.4
Whole cells	A	1.7	1.4	14.2	21.1	57.3
	B	12.4	5.5	25.6	31.3	24.9

The amount of radioactivity bound to PBPs was determined by scanning the fluorograms shown in Figs 1 and 2A and B with a Joyce-Loebl microdensitometer and calculating the area of the peaks thus obtained. The amount of radioactivity bound to each PBP is expressed as per cent of the total radioactivity bound. A, Cells grown at 32 °C; B, cells grown at 45 °C.

penicillin was unaffected by the temperature (32, 37 or 45 °C) used during the binding assay (data not shown). Finally, PBPs of membranes from cells grown at 32 °C in streptococcal broth (SB)⁵, a condition allowing a generation time of 35 min, appeared grossly different from those observed in membranes from cells grown at the same temperature in CDM and rather similar to those from cells grown in CDM at 45 °C (Fig. 1). Thus, two different conditions (temperature of incubation and growth medium) associated with changes in growth rate of the cells are also accompanied by changes in the physiological status of PBPs.

Cells growing exponentially at 32 and 45 °C were then treated with different concentrations of ^{14}C -benzylpenicillin and PBPs detected by polyacrylamide gel electrophoresis and fluorography. It is evident from Fig. 2 that the relative amount of radioactivity bound to the various PBPs of cells growing at different rates is also different when tested by *in vivo* binding experiments. Note that the differences observed in these conditions, although not similar for all proteins (compare PBPs 1 and 3), were most often of the same type as seen in isolated membranes (see Table 1). The differences between membranes and whole cells could be due to the different accessibility of some PBPs in whole cells and isolated membranes. Alternatively, they could depend on the incomplete saturation of PBPs in whole cells.

Concerning identification of the PBP that could be the lethal target of penicillin, Fig. 2 shows that only PBP 3 binds the antibiotic in cells growing at 45 °C in the presence of 0.04 $\mu\text{g ml}^{-1}$ of ^{14}C -benzylpenicillin, a concentration corresponding to the MIC. In cells growing at 32 °C and in the presence of the above concentration of antibiotic, PBP 3 was again the only protein that bound radioactivity. However, in these cells, the radioactivity bound to PBP 3 was approximately fourfold lower

Table 2 Evaluation of the absolute amount of radioactivity bound to PBPs in cells growing in the presence of various ^{14}C -benzylpenicillin concentrations

^{14}C -benzylpenicillin concentration ($\mu\text{g ml}^{-1}$)		Area of the peak (mm^2) corresponding to				
		PBP 1	PBP 2	PBP 3	PBP 4	PBP 6
0.02	A	—	—	43	—	—
	B	—	—	100	—	—
0.04	A	—	—	53	—	—
	B	—	—	228	—	—
0.08	A	—	11	82	—	—
	B	48	40	252	—	—
0.32	A	6	15	112	32	54
	B	72	48	274	—	21
0.80	A	32	18	189	297	580
	B	105	57	280	324	63
4.00	A	25	20	200	352	804
	B	161	72	332	406	322

The intensities of the bands were measured by scanning the fluorograms shown in Fig. 2A and B and calculating the area of the peaks thus obtained. A, Cells growing at 32 °C; B, cells growing at 45 °C.

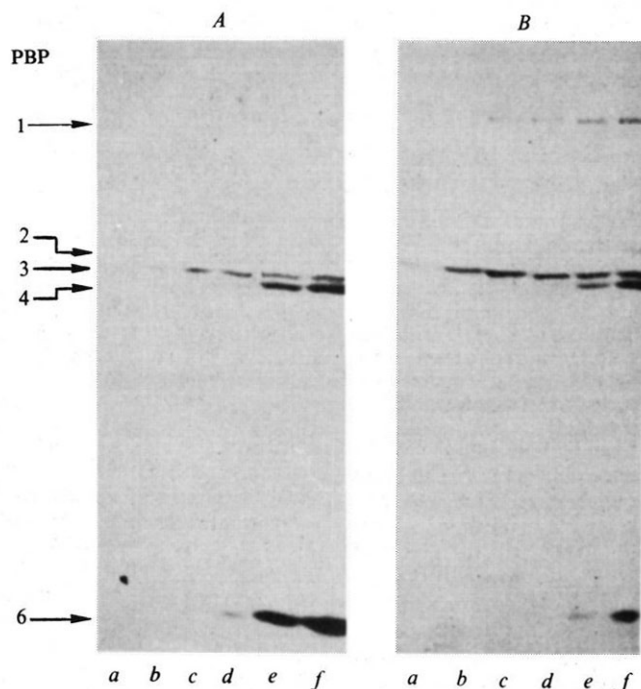


Fig. 2 Penicillin binding to membrane proteins in whole cells of *S. faecalis*. Samples (10 ml) of cells exponentially growing at 32 (A) or 45 °C (B) (2×10^8 bacteria per ml) were treated with doses of ^{14}C -benzylpenicillin ranging from 0.02 to 4 $\mu\text{g ml}^{-1}$. After 5 min, 10 mM cold penicillin was added, the cultures were rapidly chilled and membranes from cells were prepared as described previously⁵ with the only exception that lysozyme digestion was carried out by incubating the cells for 30 min at the same temperature as used for growth. No significant breakdown of PBP-penicillin complexes occurred during the preparation of membranes, as only PBP 4 showed a 10% loss of the radioactive label after 30 min of incubation at either temperature (data not shown). Electrophoresis of radioactive membranes and detection of PBPs were carried out as described previously^{13,14}. The following concentrations of ^{14}C -benzylpenicillin were used ($\mu\text{g ml}^{-1}$): a, 0.02; b, 0.04; c, 0.08; d, 0.32; e, 0.8; f, 4.

than that bound in cells growing at 45 °C in the same conditions (Table 2). In the presence of the minimal concentration of benzylpenicillin causing inhibition of cell growth at 32 °C, all PBPs bound radioactivity and the amount of ^{14}C -benzylpenicillin bound by PBP 3 was very close to that bound in the presence of the MIC at 45 °C (Fig. 2, Table 2).

The data presented here clearly indicate that growth of cells incubated at 45 °C could be inhibited through specific binding of penicillin to PBP 3. This protein seems to be the lethal target in *S. faecalis* and sensitivity to the antibiotic seems to depend on the capability of penicillin to bind it. Furthermore, (1) penicillin binds only to PBP 3 in cells growing at 45 °C in the presence of the MIC of the antibiotic; (2) in cells which require a much higher dose of antibiotic for inhibition, a much reduced amount of radioactivity is bound to PBP 3; (3) in the presence of the respective MIC of penicillin at both 32 and 45 °C, the same amount of radioactivity is bound to PBP 3. This could suggest that a minimal dose of penicillin is required to bring about interference with the vital function of PBP 3. Spratt has recently described a mutant of *Escherichia coli* in which resistance to mecillinam is due to the reduced capability of PBP 2 to bind the antibiotic². Similarly, the reduced sensitivity to penicillin of *S. faecalis* observed at 32 °C could be due to the reduced amount of penicillin bound by PBP 3.

Alternatively, the reduced sensitivity observed at 32 °C could be due to the fact that PBP 3 cannot be the lethal target in cells growing in these conditions. This possibility is supported by the fact that the amount of penicillin bound to other PBPs also changes in these conditions. PBPs could perform their functions

by interacting in a complex way so that the function of each one (or some of them) depends on the activity of other PBPs (see also ref. 6). In *S. faecalis*, PBP 3 could have an essential role in cell growth at 45 °C, but not at 32 °C, due to changes in the physiological status of this and other PBPs. This could explain different effects observed by various authors and the different mechanisms of bacterial killing proposed⁷⁻¹². Penicillins could have the main property of binding to proteins and interfering with their functions. The effects of penicillins could then depend on the specific function of the bound proteins in a given system and in specific experimental conditions.

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1. Blumberg, P. M. & Strominger, J. L. *Bact. Rev.* **38**, 291-335 (1974).
2. Spratt, B. G. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2999-3003 (1975); *Nature* **274**, 713-715 (1978).
3. Reynolds, P. E., Shepherd, S. T. & Chase, H. A. *Nature* **271**, 568-570 (1978).
4. Shockman, G. D. in *Analytical Microbiology* (ed. Kavanagh, F.) 567 (Academic, New York, 1962).
5. Coyette, J., Ghuyssen, J. M. & Fontana, R. *Eur. J. Biochem.* **88**, 297-305 (1978).
6. Giles, A. F. & Reynolds, P. E. *Nature* **280**, 167-168 (1979).
7. Satta, G., Canepari, P., Botta, G. & Fontana, R. *J. Bact.* **142** (1980).
8. Wise, E. M. & Park, J. T. *Proc. natn. Acad. Sci. U.S.A.* **54**, 75-81 (1965).
9. Tipper, D. J. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **54**, 1133-1141 (1965).
10. Hammes, W. P. *Eur. J. Biochem.* **70**, 107-113 (1976).
11. Tomasz, A. & Waks, S. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4162-4166 (1975).
12. Fontana, R., Satta, G. & Romanzi, C. A. *Antimicrob. Ag. Chemother.* **12**, 745-747 (1977).
13. Laemli, U. K. & Favre, M. *J. molec. Biol.* **80**, 575-599 (1973).
14. Bonner, W. M. & Laskey, R. A. *Eur. J. Biochem.* **46**, 83-88 (1974).

***In vitro* tumorigenic transformation by a defined sub-genomic fragment of bovine papilloma virus DNA**

Douglas R. Lowy*, Israel Dvoretzky*, Ralph Shober†, Ming-Fan Law‡, Linda Engel‡ & Peter M. Howley‡

* Dermatology Branch,

† Laboratory of Tumor Virus Genetics,

‡ Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20205

The papillomaviruses, which are widely distributed in nature, typically induce benign skin tumours (warts) in their natural hosts (reviewed in ref. 1). These viruses are members of the papovavirus group, as are the polyomaviruses². In contrast to polyomaviruses such as SV40, polyoma and BK (reviewed in ref. 3), the papillomaviruses have not been extensively studied, primarily because of the lack of a suitable tissue culture system. In general, the proliferative skin changes induced by the papillomaviruses are limited to the epidermal cells, and the viruses have a very narrow host range. However, some bovine papilloma viruses (BPV) are exceptional in that they induce fibropapillomas in their natural host (cows); these BPV can also induce cellular transformation in tissue culture⁴⁻⁶ and fibroblastic tumours in heterologous animals, including hamsters⁷, mice⁸ and horses^{8,9}. BPV DNA from virions can also transform rodent cells and is tumorigenic in hamsters¹⁰. Recently, a tissue culture focus assay¹¹ in mouse cells (NIH 3T3 and C127) has been described for those BPV which are the aetiological agents of fibropapillomas in cows. The virally transformed cells, which did not contain infectious virus, grew in soft agar and were tumorigenic in nude (*nu/nu*) mice. We¹² have recently molecularly cloned the entire genomes of BPV-1 and BPV-2 (ref. 13) in *Escherichia coli* K12 using the certified plasmid vector pBR322 (ref. 14). We now demonstrate that these cloned DNAs can transform NIH 3T3 and C127 cells. Further, a molecularly cloned fragment which contains 69% of the BPV-1 genome can also induce cellular transformation.

A linear physical map of the 8-kilobase pair closed circular BPV-1 DNA genome (from papilloma 307) is shown at the top of Fig. 1. Restriction endonucleases *Hind*III, *Bam*HI, *Eco*RI and *Hpa*I each cleave the viral DNA once, at 0, 0.31, 0.60 and 0.88 map units, respectively¹⁵. The BPV DNA genome was cloned in pBR322 at the *Bam*HI site (clone 8-2) and also at the *Hind*III site (clone 9-1)¹². After digestion with the enzyme used to insert BPV DNA into the pBR322 vector, the DNA was transfected¹⁶ into C127 and NIH 3T3 mouse cells. Within 2 weeks, foci of transformed cells appeared in each cell line (Fig. 1, expt 1). These foci, which were more distinct on the C127 cells than in the NIH 3T3 cells, were indistinguishable morphologically from foci induced by BPV virions in the same cells. Transfection of NIH 3T3 cells resulted in two to three times more foci than observed in C127 cells with either BPV-1 cloned DNA; this greater sensitivity of the NIH 3T3 cells was also noted following infection with whole virions¹⁰. In each cell line the two different BPV-1 DNA preparations induced a similar number of foci. BPV-2 DNA, which shows extensive sequence homology with BPV-1 DNA, was cloned in pBR322 at its unique *Hind*III site; after digestion with *Hind*III, this cloned DNA also induced foci in this transfection experiment (data not shown).

Because BPV-1 DNA linearized at either the *Hind*III or *Bam*HI site was equally efficient in transforming these mouse cells, it seemed likely that some BPV-1 sequences might not be required for the induction of cellular transformation. We therefore examined the ability of defined sub-genomic fragments of the cloned viral DNAs to transform the mouse cells. The DNA fragments were prepared by digestion of the *Bam*HI BPV-1 DNA insert with a second restriction endonuclease which cleaved the BPV DNA at a unique site (*Hind*III, *Eco*RI and *Hpa*I). The BPV fragments were separated by agarose gel electrophoresis, individually eluted from the gel and transfected into the mouse cells. Of the various cleavage products tested, only the 69% *Bam*HI/*Hind*III fragment still induced foci, although its efficiency was lower than that of the complete BPV genome (expt 2). The other sub-genomic fragments tested failed to induce foci.

To eliminate the possibility that the infectivity of the 69% *Bam*HI/*Hind*III fragment might be due to the transfection of additional BPV DNA sequences contaminating this DNA in the

Table 1 Growth in agar and tumorigenicity of BPV DNA transformants

Transforming agent	Colony formation in agar	Tumour formation in nude mice
Control cells	0	0/4
BPV-1 virus	1.4×10^3 *	4/4†
<i>Hind</i> III genome, focus 52	0.6×10^3	4/4
<i>Bam</i> HI genome, focus 33	2.2×10^3	4/4
<i>Bam</i> HI genome, focus 43	1.3×10^3	4/4
69% Fragment clone 17-6, focus 21	0.8×10^3	4/4
69% Fragment clone 17-6, focus 22	1.6×10^3	4/4
69% Fragment clone 17-2, focus 24	1.1×10^3	4/4
69% Fragment clone 17-2, focus 25	1.9×10^3	4/4

Each BPV DNA transformant was isolated from a different dish of C127 cells to ensure that each was separately derived. Each focus of transformed cells was picked 3 weeks after transfection of DNA and propagated separately. Colony formation in 0.35% agar was carried out as described elsewhere¹⁸. Weanling nude mice were inoculated subcutaneously with 10^6 transformed cells. Tumours were present in all animals by 3 weeks after inoculation. Control animals were observed for 8 weeks.

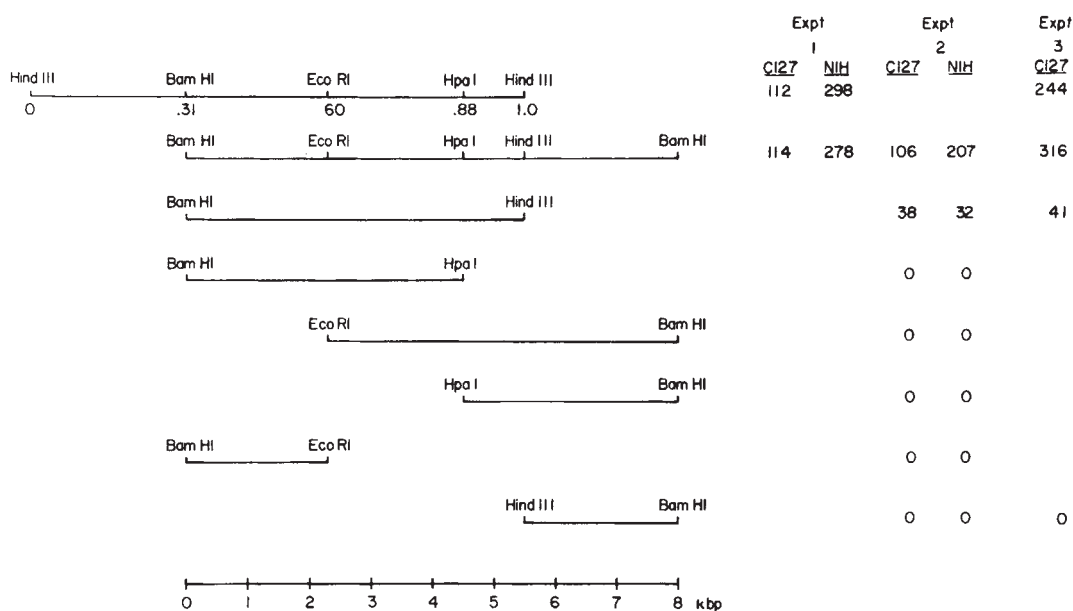
* No. of colonies per 10^4 cells.

† No. of mice with tumours/no. of mice inoculated with cells.

agarose gel, this fragment and the 31% *Bam*HI/*Hind*III fragment were each subcloned in pBR322 from the *Bam*HI BPV clone. Two subclones of each fragment were tested in the C127 mouse cell transfection assay. After separating the BPV DNA from the pBR DNA by digestion with *Bam*HI/*Hind*III, both the 69% fragment clones induced foci but the 31% fragment clones failed to do so (expt 3). As noted previously, the specific infectivity of the 69% sub-genomic fragment was lower than that of the complete viral genome. Southern blot analysis of DNA from the transformed cells has indicated that, as expected, the transformants contained BPV DNA sequences from the 69% fragment and no sequences which hybridized with the 31% fragment (M.-F.L. *et al.*, unpublished data).

A good correlation has been noted¹⁷ between anchorage-independent growth of transformed cells and their capacity to be tumorigenic in nude mice. The ability of the BPV-transformed

Fig. 1 Duplicate dishes were transfected with restriction endonuclease-digested DNA (0.1 to 0.5 μ g per dish) using calf thymus DNA (25 μ g ml⁻¹) as carrier^{16,24} (similar results were obtained when NIH 3T3 DNA was used as carrier). Foci were counted at 2 weeks for C127 cells and 4 or 5 days later for NIH 3T3 cells. Results are expressed as focus forming units per μ g BPV DNA. pBR322 controls were included with each experiment and did not induce foci. A zero means that no foci were observed after testing 1 μ g of DNA. In expt 2, the *Bam*HI DNA insert from clone 8-2 was digested with each of the other three enzymes. The DNA



was electrophoresed in a 1% agarose gel after restriction endonuclease digestion. The ethidium bromide-stained BPV DNA bands were cut out of the gel and transfected into the cells as previously described²⁴. In expt 3, the 69% and 31% *Bam*HI/*Hind*III fragments used were clones 17-6 and 16-9, respectively. The DNAs were digested with *Hind*III/*Bam*HI, electrophoresed and transfected as in expt 2. kbp, Kilobase pairs.

cells to form colonies in agar¹⁸ and to induce tumours in weanling nude mice has therefore been tested. Transformants derived from individual foci induced in C127 cells by the *Hind*III BPV genome, the *Bam*HI BPV genome and the 69% *Bam*HI/*Hind*III fragment all grew in soft agar; these cells also induced tumours in the mice after a latent period of 2–3 weeks (Table 1). Mice inoculated with control cells did not develop tumours during 8 weeks of observation.

These observations indicate that not all sequences of the BPV genome are required for cellular transformation. In this regard, the results are similar to those reported for other DNA tumour viruses, such as polyomaviruses and adenoviruses (reviewed in ref. 19). The sequences contained in the 31% *Bam*HI/*Hind*III fragment were not essential for the induction of transformation. It is likely that further study may reveal additional BPV sequences which are not required for transformation.

It is not clear why the transforming efficiency of the subgenomic fragment was lower than that of the entire viral genome. It is possible that some of the BPV sequences from 0–0.31 map units may have a specific facilitative role, as has been shown in transformation^{20–22} by retroviruses. Alternatively, the smaller size of the fragment or the presence of non-homologous ends may introduce physical constraints which by themselves lower the efficiency of the transfection assay, if circularization of the viral DNA occurs. These or other possibilities may be evaluated by future studies.

The precise relevance of these *in vitro* results to *in vivo* tumours induced by BPV or other papillomaviruses remains to be established. From our data, it may be speculated that *in vivo* transformation of epidermal and fibroblastic cells by BPV does not depend on viral replication. This hypothesis is supported by the observation that the fibroblastic component of BPV-induced tumours does not contain detectable amounts of infectious virus⁸. Some data also suggest that the results obtained with BPV may be relevant to infection with other papillomaviruses. It has recently been demonstrated²³ that BPV DNA (types 1 and 2) contains nucleotide sequences which show homology with human papilloma virus type 1 and with Shope rabbit papilloma virus; this suggests that these viral genomes may be organized similarly. It may further be significant that one of these regions of homology is contained within the transforming 69% *Bam*HI/*Hind*III BPV-1 DNA fragment.

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- Orth, G., Breitburd, F., Favre, M. & Croissant, O. in *Origins of Human Cancer* (eds Hiatt, H. H., Watson, J. D. & Wisten, J. A.) 1043–1068 (Cold Spring Harbor Laboratory, New York, 1977).
- Melnick, J. L. *et al.* *Intervirology* **3**, 106–120 (1974).
- Howley, P. M. in *Viral Oncology* (ed. Klein, G.) 489–550 (Raven, New York, 1980).
- Black, P. H., Hartley, J. W., Rowe, W. P. & Huebner, R. J. *Nature* **199**, 1016–1018 (1963).
- Boiron, M., Levy, J. P., Thomas, M., Friedman, J. C. & Bernard, J. *Nature* **201**, 423–424 (1964).
- Thomas, M., Boiron, M., Tanzer, J., Levy, J. P. & Bernard, J. *Nature* **202**, 709–710 (1964).
- Friedman, J. C. *et al.* *C. r. heb. Séanc. Acad. Sci., Paris* **257**, 2328–2331 (1963).
- Olson, C., Gordon, D. E., Robl, M. G. & Lee, K. P. *Archs envir. Hlth* **19**, 823–839 (1969).
- Lancaster, W. D., Olson, C. & Meinke, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 524–528 (1977).
- Boiron, M., Thomas, M. & Chénaille, P. *Virology* **26**, 150–153 (1965).
- Dvoretzky, I., Shober, R., Chattopadhyay, S. K. & Lowy, D. R. *Virology* **103**, 369–375 (1980).
- Howley, P. M. *et al.* in *Viruses in Naturally Occurring Cancers* (eds Essex, M. *et al.*) (Cold Spring Harbor Laboratory, New York, in the press).
- Lancaster, W. D. & Olson, C. *Virology* **89**, 372–379 (1978).
- Bolivar, F. *et al.* *Gene* **2**, 95–113 (1977).
- Lancaster, W. D. *J. Virol.* **32**, 684–687 (1979).
- Graham, F. J. & van der Eb, A. J. *Virology* **52**, 456–461 (1973).
- Shin, S., Freedman, V. H., Risser, R. & Pollack, R. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4435–4439 (1975).
- MacPherson, I. & Montagnier, L. *Virology* **23**, 291–294 (1964).
- Graham, F. L. *et al.* *Cold Spring Harb. Symp. quant. Biol.* **39**, 637–650 (1974).
- Andersson, P., Goldfarb, M. P. & Weinberg, R. A. *Cell* **16**, 63–75 (1979).
- Oskarsson, M., McClements, W. L., Blair, D. L., Vande Woude, G. F. & Maizel, J. V. *Science* **207**, 1222–1224 (1980).
- Chang, E. H. *et al.* *J. Virol.* **35**, 76–92 (1980).
- Law, M.-F., Lancaster, W. D. & Howley, P. M. *J. Virol.* **32**, 199–207 (1979).
- Lowy, D. R., Rands, E. & Scolnick, E. M. *J. Virol.* **26**, 291–298 (1978).

Calcium-dependent phosphorylation of histone H3 in butyrate-treated HeLa cells

James P. Whitlock Jr, Robert Augustine & Howard Schulman

Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

Ca^{2+} is prominent in the control of cell proliferation and function^{1,2}. However, the biochemical mechanism(s) mediating its effects on nuclear events is unknown. We report here that Ca^{2+} , at physiological concentrations, stimulates the phosphorylation of histone H3 by an endogenous protein kinase in HeLa cell nuclei. Also, pretreatment of cells with Na butyrate, which increases histone acetylation, selectively increases the susceptibility of histone H3 to phosphorylation by the protein kinase. Our results reveal a potential link between histone H3 acetylation and phosphorylation, modifications which are thought to have important effects on chromatin structure and function and suggest a possible mechanism whereby stimuli at the cell surface (such as hormones, mitogens and drugs) may influence biochemical events at the nuclear level; changes in the intracellular Ca^{2+} concentration may influence the phosphorylation of chromosomal proteins, mediated by Ca^{2+} -dependent kinases in the nucleus.

HeLa cells are known to contain a nuclear kinase(s) which phosphorylates histone H3 (ref. 3). We studied histone phosphorylation by incubating HeLa nuclei with [γ -³²P]ATP. The nuclei were then washed in 0.3 M NaCl to remove many of the non-histone nuclear proteins. The salt-washed nuclei were extracted with dilute HCl, and the acid-soluble proteins were precipitated and analysed by SDS-polyacrylamide gel electrophoresis and autoradiography. The autoradiogram shown in Fig. 1 indicates that radioactive phosphate is incorporated into an acid-soluble protein which has a mobility identical to that of histone H3. Additional experiments (not shown) indicate that this protein is not removed when nuclei are washed in 0.6 M NaCl but is removed when the NaCl concentration is raised to 2.0 M. Thus, the properties of this protein are identical to those of histone H3 (ref. 4).

Ca^{2+} , at submicromolar concentrations, causes a marked and selective stimulation of ³²P incorporation into histone H3 (Fig. 1). In these experiments, the free Ca^{2+} concentration was varied by using a Ca^{2+} -EGTA buffer containing 0.4 mM EGTA (ref. 5). Quantitative densitometry of the autoradiograms indicates that Ca^{2+} , at concentrations of 0.1 μM and 0.5 μM , increases the phosphorylation of histone H3 two- to threefold. Higher Ca^{2+} concentrations (up to 10 mM) produce no further increase in H3 phosphorylation (data not shown). Thus, the phosphorylation of histone H3 is stimulated by Ca^{2+} at concentrations which are presumably physiological. In analogous experiments, we observed that cyclic AMP (10 μM) and cyclic GMP (10 μM) had no effect on H3 phosphorylation (data not shown).

The amino acid residues in histone H3 which are known to undergo phosphorylation are located in the amino terminal region of the protein⁶; this is the same region which undergoes acetylation *in vivo*⁶. We therefore wondered whether one type of histone H3 modification (acetylation) influenced the susceptibility of the protein to a second type of modification (phosphorylation). Cells cultured in the presence of butyric acid accumulate hyperacetylated histones, due to the inhibition of histone deacetylation by butyrate^{7–13}. In agreement with the observations of others, we find that exposure of HeLa cells to 5 mM Na butyrate for 16–24 h produces marked acetylation of histones, as judged by the distribution of acetylated forms of

Fig. 1 Calcium-dependent phosphorylation of histone H3 in HeLa cells: *a*, stained gel; *b*, autoradiogram. HeLa cells were grown in monolayers to approximate confluence in Dulbecco's modified Eagle's medium (Gibco). Cells were washed twice with ice-cold phosphate-buffered saline (PBS), collected by scraping into PBS, and pelleted. Nuclei were prepared by Dounce homogenization in the presence of 1% Triton X-100 (ref. 21). Washed nuclei were suspended in a solution of 0.15 M NaCl, 5 mM MgCl₂, 5 mM dithiothreitol and 5 mM Na butyrate in 50 mM Tris-HCl, pH 7.5 containing the indicated concentration of free Ca²⁺. The free Ca²⁺ concentration was calculated using an apparent binding constant for Ca²⁺-EGTA of $7.61 \times 10^6 \text{ M}^{-1}$ (ref. 22). The reaction was started by the addition of [γ -³²P]ATP (Amersham; 10–50 Ci mmol⁻¹) to a final concentration of 1 μM . Nuclei were incubated at room temperature (22 °C) for 5 min. Preliminary experiments indicated that, under these conditions, the phosphorylation of histone H3 continues to completion. The phosphorylation reaction was stopped by the addition of EDTA to a final concentration of 10 mM. Nuclei were washed following the addition of NaCl to a final concentration of 0.3 M and the salt-washed nuclei were collected by centrifugation. The salt-washed nuclei were extracted for 1 h with ice-cold 0.25 M HCl and centrifuged to pellet the precipitate. The supernatant was removed and precipitated overnight at –20 °C by the addition of 10 volumes of acetone. The precipitate was collected by centrifugation, dissolved in sample buffer and analysed by electrophoresis in discontinuous SDS-polyacrylamide gels²³. Purified calf thymus histone H2B (Boehringer-Mannheim) was phosphorylated *in vitro*, using [γ -³²P]ATP and a purified cyclic AMP-dependent protein kinase and was included in the analysis as a molecular weight standard. The gels were stained with Coomassie blue, destained, and subjected to autoradiography as previously described^{23,24}. Electrophoresis was from top to bottom. The positions of the core histones are indicated. Std, H2B standard.

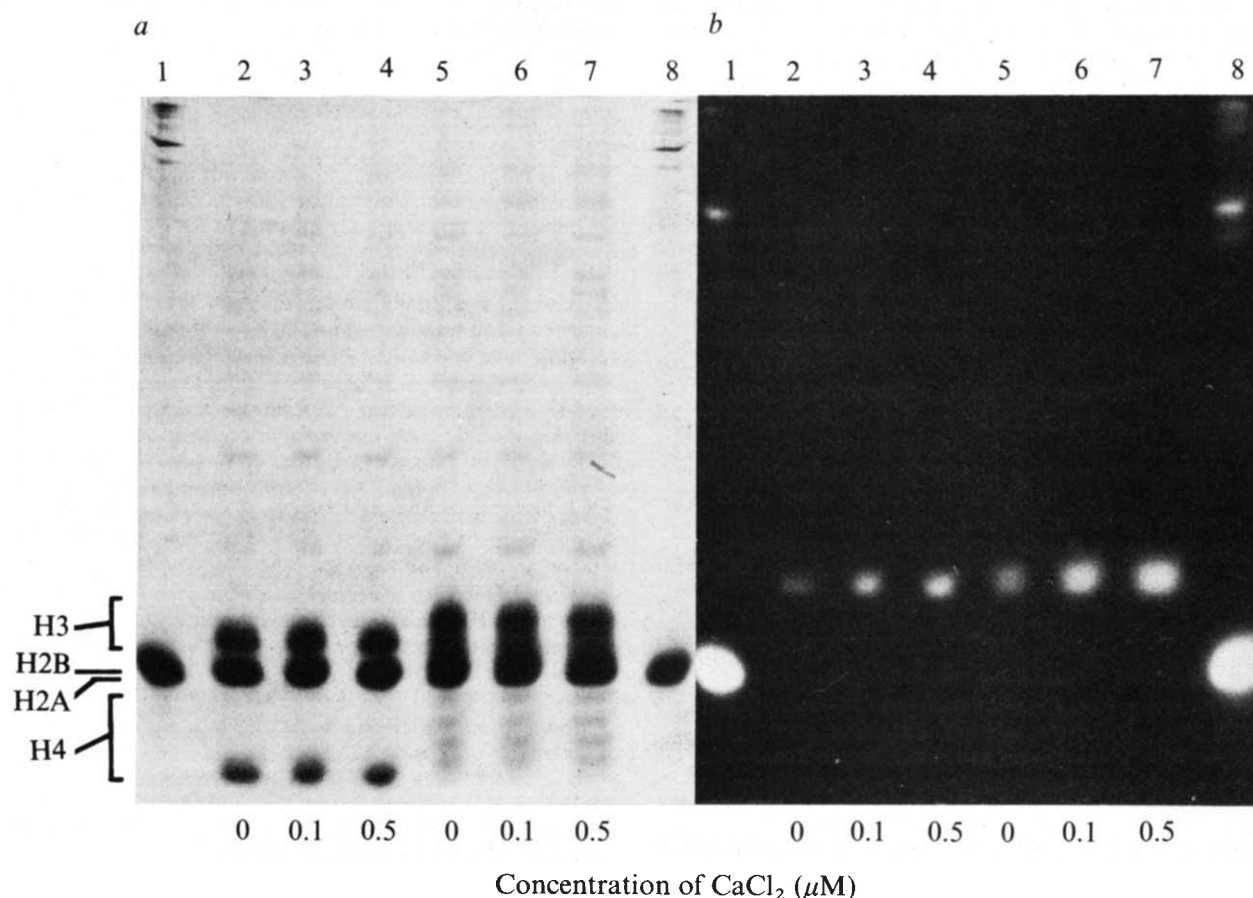
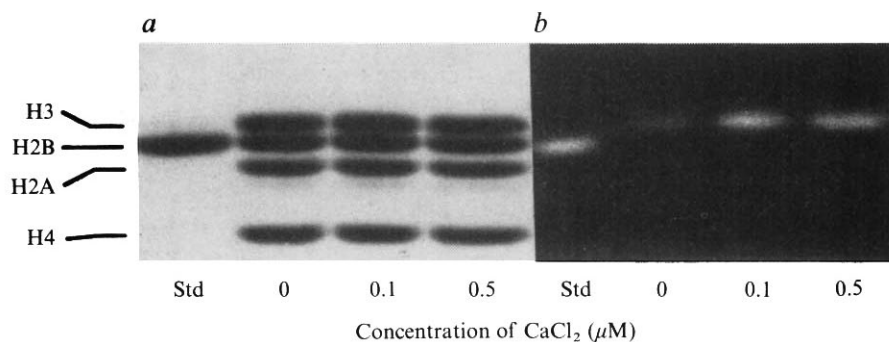


Fig. 2 Histone H3 phosphorylation in butyrate-treated HeLa cells: *a*, stained gel; *b*, autoradiogram. Cells were incubated for 16 h in the absence or presence of Na butyrate (5 mM). Cells were collected and nuclei were prepared as described in the legend to Fig. 1, except that, in the case of butyrate-treated cells, all solutions contained 5 mM Na butyrate. The phosphorylation reaction and isolation of acid-soluble proteins was as described in the legend to Fig. 1. The acetone precipitate was dissolved in sample buffer and analysed by electrophoresis in polyacrylamide gels containing acetic acid and urea¹². Gels were stained in Coomassie blue, destained, and subjected to autoradiography as previously described^{23,24}. Electrophoresis was from top to bottom. The positions of the core histones are indicated. Lanes 1 and 8, H2B standard; lanes 2–4, control (not butyrate-treated); lanes 5–7, butyrate-treated.

histone H4 after electrophoresis in polyacrylamide gels containing acetic acid and urea.

We prepared nuclei from control and butyrate-treated cells (5 mM, 16 h), incubated them in the presence of [γ - 32 P]ATP, washed the nuclei in 0.3 M NaCl, extracted the nuclei with dilute HCl, and analysed the acid-soluble protein by electrophoresis in acetic acid-urea-polyacrylamide gels, followed by autoradiography. The results (Fig. 2) indicate that histone H3 in butyrate-treated cells is more susceptible to phosphorylation than is histone H3 from control cells. Quantitative densitometry of the autoradiogram indicates that two to three times as much phosphate is incorporated into histone H3 in butyrate-treated cells as is incorporated into histone H3 in control cells (data not shown). In both control and butyrate-treated cells, the phosphorylation of histone H3 is stimulated two- to threefold in the presence of submicromolar concentrations of Ca^{2+} (Fig. 2).

Several kinds of experiments suggest that it is acetylation, *per se*, which renders histone H3 more susceptible to phosphorylation: (1) dose-response experiments (exposure of cells to 0–50 mM Na butyrate for 16 h) and time-course experiments (5 mM Na butyrate for 0–24 h) demonstrate a correlation between increased acetylation and increased phosphorylation; (2) both the hyperacetylation and the increased susceptibility to phosphorylation are rapidly reversed following the removal of butyrate (5 mM) from the medium; (3) exchange experiments (such as incubating kinase-depleted nuclei prepared from butyrate-treated cells with protein kinase activity prepared from control nuclei and vice versa) indicate that the increased phosphorylation is due to an alteration in the histone substrate rather than to a change in the kinase (data not shown).

Our results suggest that an acetylated form(s) of histone H3 is particularly susceptible to phosphorylation and that this event is Ca^{2+} -dependent. The effect of Ca^{2+} presumably reflects a stimulation of protein kinase activity, although we cannot rule out the possibility that its primary effect is to alter the conformation of the histone substrate. The absence of a stained band with a mobility identical to that of the phosphorylated H3 species indicates that it is a relatively small subpopulation of H3 molecules which becomes phosphorylated (Fig. 2). The autoradiogram (Fig. 2) indicates that the phosphorylated form(s) of histone H3 migrates more slowly than the most highly acetylated form(s) of H3 visible on the stained gel. Our findings are consistent with the idea that, in both control and butyrate-treated cells, the most highly acetylated form(s) of H3 is most susceptible to phosphorylation.

In eukaryotic cells, the arginine-rich histones H3 and H4 seem to be of fundamental importance in the folding of nuclear DNA into the nucleoprotein complex known as chromatin^{14–16}. The amino acid sequences of both H3 and H4 have been highly conserved throughout evolution¹⁷; therefore, it is argued that, in principle, chemical modification of a histone(s) could produce important changes in chromatin structure and function. In practice, there is considerable circumstantial evidence to support the view that both acetylation and phosphorylation of the histones have substantial structural and functional effects on the nucleoprotein^{6,14,17–19}.

Recently, there has been renewed appreciation of the importance of Ca^{2+} in regulating various cellular functions (such as the response to hormonal stimulation and the stimulus-coupled release of neurotransmitters or peptide hormones)^{1,2}. It is becoming apparent that Ca^{2+} -dependent protein kinases may mediate some of the intracellular actions of Ca^{2+} and thereby play a central role in regulating the physiological responses of the cell to changes in its external environment²⁰. Such Ca^{2+} -dependent kinases have been identified in both membrane and cytosol preparations. Our experiments indicate that such an enzyme(s) may also be present in the nucleus and can utilize a chromosomal protein as a substrate, suggesting a mechanism by which changes in intracellular Ca^{2+} may influence biochemical events in the cell nucleus.

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1. Berridge, M. J. in *Advances in Cyclic Nucleotide Research* Vol. 11 (eds Greengard, P. & Robison, G. A.) 1–98 (Raven, New York, 1975).
2. Rasmussen, H. & Goodman, D. B. P. *Physiol. Rev.* **57**, 421–509 (1977).
3. Simpson, R. T. *Nucleic Acids Res.* **5**, 1109–1119 (1978).
4. Hnilica, L. S. *The Structure and Biological Function of Histones* (Chemical Rubber Co., Cleveland, 1972).
5. Schulman, H. & Greengard, P. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5432–5436 (1978).
6. Dixon, G. H. et al. *Ciba Fdn Symp.* **28** (new series), 229–250 (1975).
7. Riggs, M. G., Whittaker, R. G., Newmann, J. R. & Ingram, V. M. *Nature* **268**, 462–464 (1977).
8. Boffa, L. C., Vidali, G., Mann, R. S. & Allfrey, V. G. *J. biol. Chem.* **253**, 3364–3366 (1978).
9. Vidali, G., Boffa, L. C., Bradbury, E. M. & Allfrey, V. G. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2239–2243 (1978).
10. Candido, E. P. M., Reeves, R. & Davie, J. R. *Cell* **14**, 105–113 (1978).
11. Sealy, L. & Chalkley, R. *Cell* **14**, 115–121 (1978).
12. Cousens, L. S., Gallwitz, D. & Alberta, B. M. *J. biol. Chem.* **254**, 1716–1723 (1979).
13. Panyim, S. & Chalkley, R. *Archs Biochem. Biophys.* **130**, 337–346 (1969).
14. Elgin, S. C. R. & Weintraub, H. A. *Rev. Biochem.* **44**, 725–774 (1975).
15. Kornberg, R. D. A. *Rev. Biochem.* **46**, 931–954 (1977).
16. Felsenfeld, G. *Nature* **271**, 115–122 (1978).
17. Isenberg, I. A. *Rev. Biochem.* **48**, 159–191 (1979).
18. Gurley, L. R., D'Anna, J. A., Barkham, S. S., Deaven, L. L. & Tobey, R. A. *Eur. J. Biochem.* **84**, 1–15 (1978).
19. Langan, T. A. *Meth. Cell Biol.* **19**, 127–142 (1978).
20. Schulman, H. in *Handbook of Experimental Pharmacology* (eds Nathanson, J. A. & Keibabian, J. W.) (Springer, Berlin, in the press).
21. Whitlock, J. P. Jr & Simpson, R. T. *Biochemistry* **15**, 3307–3314 (1976).
22. Portzehl, H., Caldwell, P. C. & Reugg, J. C. *Biochim. biophys. Acta* **79**, 581–591 (1964).
23. Whitlock, J. P. Jr *J. biol. Chem.* **254**, 5684–5689 (1979).
24. Simpson, R. T. & Whitlock, J. P. Jr *Cell* **9**, 347–353 (1976).

Acetylation of histone H4 and its role in chromatin structure and function

S. S. Chahal*, H. R. Matthews†‡ & E. M. Bradbury†

* Portsmouth Polytechnic, Biophysics Laboratories, St Michael's Building, White Swan Road, Portsmouth, Hampshire PO1 2DT, UK
† Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616

Histone H4 is a highly conserved structural component of the nucleosome subunit of chromatin. The activation of chromatin is accompanied by changes in structure which may be caused by histone modification or by interactions of specific non-histone proteins, or both. Histone H4 can be modified by acetylation and this modification has been correlated with chromosome assembly¹ and with transcription². We have now tested these correlations by studying H4 acetate content as a function of the cell cycle using the naturally synchronous cell cycle in *Physarum polycephalum*. The results show two clear correlations: (1) tetra-acetylated H4 correlates with transcription; (2) highly acetylated H4 (2 to 4 acetates per molecule) is inversely correlated with H1 phosphorylation and initiation of chromosome condensation in prophase. The results are consistent with turnover of di-acetylated H4 during chromosome assembly in S phase.

Mohberg and Rusch³ showed that *Physarum* has histones like other eukaryotes and the bands they identified as H1 and H4 have recently been isolated⁴. The amino acid composition of *Physarum* H4 shows no significant difference from the amino acid composition of calf thymus H4. *Physarum* H4 co-migrates with calf thymus H4 in polyacrylamide gel electrophoresis (PAGE) in acetic acid-urea or SDS or Triton X-100 (Fig. 1).

Physarum H4 splits into five bands on long polyacrylamide gels with acetic acid-urea or Triton X-100 but not with SDS. Only very small amounts of 32 P are incorporated into *Physarum* H4 in plasmodia⁵—certainly not enough to account for the observed heterogeneity. By analogy with other H4 histones the heterogeneity was attributed to acetylation and this was confirmed by pulse-labelling experiments and by enhancement

‡ To whom correspondence should be addressed.

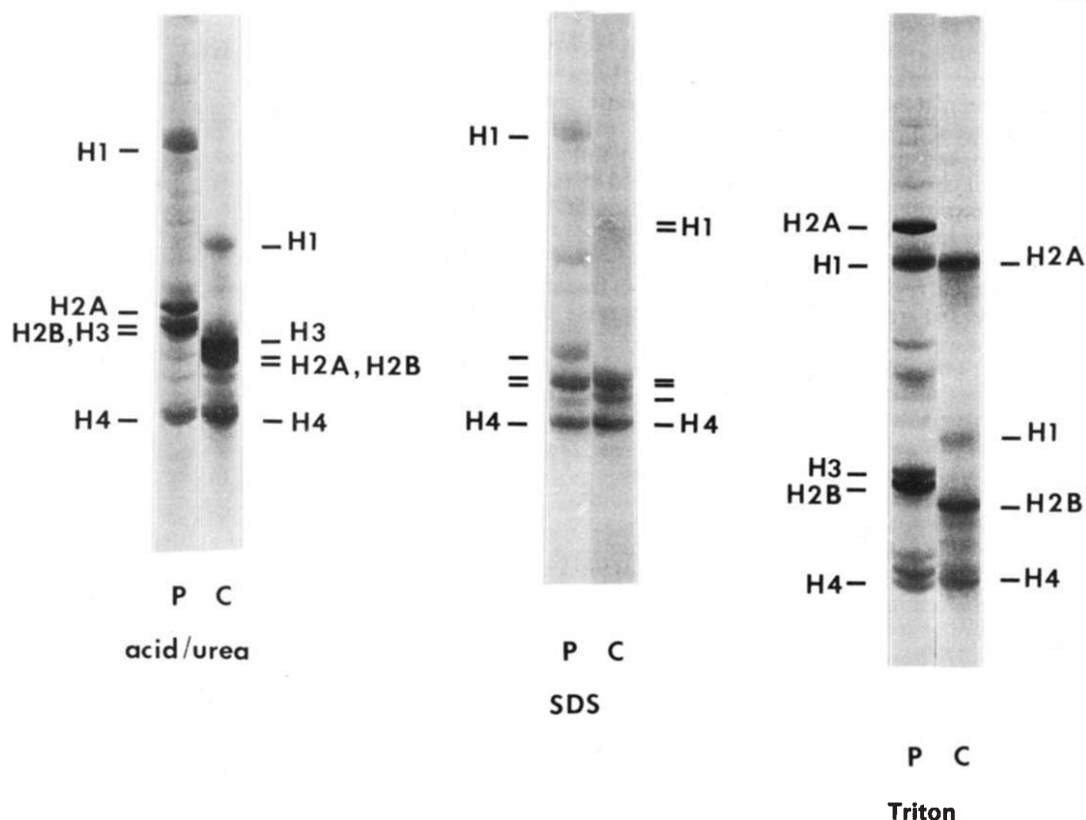


Fig. 1 Comparison of histones from *Physarum* plasmodia and calf thymus by acrylamide gel electrophoresis in the presence of SDS³⁵; acetic acid and urea⁸; Triton X-100³⁶. C, calf thymus; P, *Physarum*. The labelled bands were identified by electrophoresis of purified histone fractions. Electrophoresis was from top to bottom in 6-mm internal diameter glass tubes, 6 cm long. These gels are not long enough to resolve the H4 species adequately.

of the slower moving bands when butyrate⁶ was added to the growth medium (Fig. 2a).

The proportion of *Physarum* H4 in each of the five bands was determined by PAGE in acetic acid-urea^{7,8}. The H4 was first purified by chromatography on Biogel P10 to reduce the amount of protein loaded on the gels and to remove a minor component that interfered with the measurement of tetra-acetylated H4, Ac₄H4.

Figure 2b shows examples of gel scans of *Physarum* H4 from specific stages of the cell cycle. These and others have been analysed in terms of five gaussian peaks and the area under each peak calculated. The areas were thus fully corrected for the overlap between bands. The area corresponding to each species of H4 is plotted in Fig. 3a as a function of stage of the cell cycle.

It is clear that at all stages the most abundant species is mono-acetylated H4, Ac₁H4. During G2 phase the proportion of Ac₁H4 rises to ~62% and then falls to ~55%. The fall occurs during the middle part of G2, from 5 h to ~9 h after mitosis in the 11-h cycle. During the 6–9-h period after mitosis there are complementary increases in proportions of Ac₂H4 (16% to 23%), Ac₃H4 (2.5% to 4.3%) and Ac₄H4 (1% to 3%). The data imply an overall increase in acetylation during mid G2 phase. Late G2 is characterized by a sharp drop in Ac₄H4 (3% to 1%), followed in early prophase by a drop in Ac₃H4 (4% to 2%). Ac₂H4 drops sharply through late G2 and prophase (23% to 11%). In early prophase the overall proportion of highly acetylated H4 (2 to 4 acetates per molecule) is at its minimum value (13.5%). During late mitosis and early S phase the increase in proportion of highly acetylated H4 is balanced by drops in both non- and mono-acetylated H4 so that in mid S phase the proportion of highly acetylated H4 reaches its maximum (33.6%). In late S phase the proportion of highly acetylated H4 falls to a plateau level (about 26%) before the minimum (19.4%) in early G2 phase. The most dramatic changes in an individual H4 species occur in Ac₄H4 and these are shown on an expanded scale in Fig. 3b. There is a clear minimum (0.4%) in early prophase, a sharp maximum (3.6%) in mid S phase and a second maximum (up to 3%) in mid G2 phase.

Histone H4 is extensively modified in the cytoplasm before entry into the nucleus in duck erythroid cells⁷ and in hepatoma

cell culture⁹. Figure 3a shows a peak of Ac₂H4 in S phase nuclei that is consistent with the notion that newly synthesized H4 enters the nucleus in the di-acetyl form and is then converted to the unmodified form, which shows an increase in late S phase (M2 + 1 h to M2 + 2.5 h) following the increase in Ac₂H4 (M2 to M2 + 1 h). This suggestion is analogous to the situation in HTC cells⁹ but pulse-label experiments are required to confirm this interpretation in *Physarum*.

Transcription in *Physarum* during the cell cycle is probably biphasic^{10–13} with a very low level in mitosis, a high level in S phase, a low in early G2 and the second high in mid to late G2. Several lines of evidence suggest that the S phase peak is predominantly mRNA synthesis and the G2 phase peak is predominantly rRNA synthesis although the distinction, if correct, is not absolute (for reviews see refs 14, 15). The biphasic pattern correlates closely with the pattern of Ac₄H4 (Fig. 3) supporting the correlation between acetylated histones and transcription. About 2% of *Physarum* DNA codes for rRNA and associated spacer sequences (rDNA)^{16,17}. If the G2 phase transcription is predominantly rRNA and is associated with Ac₄H4, then the G2 phase increase in Ac₄H4 from 1% to 3% implies that most of the H4 associated with rDNA would become tetra-acetylated in G2. This prediction can be tested directly.

D'Anna *et al.*¹⁸ found low levels of H4 acetate in mitosis in CHO cells and in the present experiments the lowest values for the proportion of highly acetylated H4 also occur in mitosis, particularly in prophase, where, at M3, the proportion of Ac₄H4 falls as low as 0.4%. The absolute values at M2 are rather different from those at M3 but the direction of change in proportion is the same. The absolute difference may reflect a change in growth pattern as the plasmodium gets larger and the composition of the medium changes. These factors add a slow monotonic change underlying the larger cell-cycle-dependent changes. The amount of highly acetylated H4 in prophase is inversely correlated with phosphorylation of histone H1. As shown in Fig. 3c, the phosphate content of H1 reaches its maximum about 20 min before metaphase in *Physarum*⁵. Histone H1 also has a high phosphate content at mitosis in HeLa cells¹⁹ and CHO cells²⁰.

There is substantial circumstantial evidence linking H1 phosphorylation in late G2 phase and prophase with chromosome condensation (for review see ref. 21). Consequently, it now seems that chromosome condensation may involve the coordinated modification of H1 by phosphorylation and of H4 by deacetylation. It follows that the extension of chromosomes after mitosis would be facilitated by histone acetylation and very rapid acetylation of histone H4 immediately following metaphase is observed.

Acetylation occurs on lysines -5, -8, -12 and -16 of H4 (ref. 1). The N-terminal region of H4 is unstructured in free solution and can be released from a nucleosome core particle by salt²² or

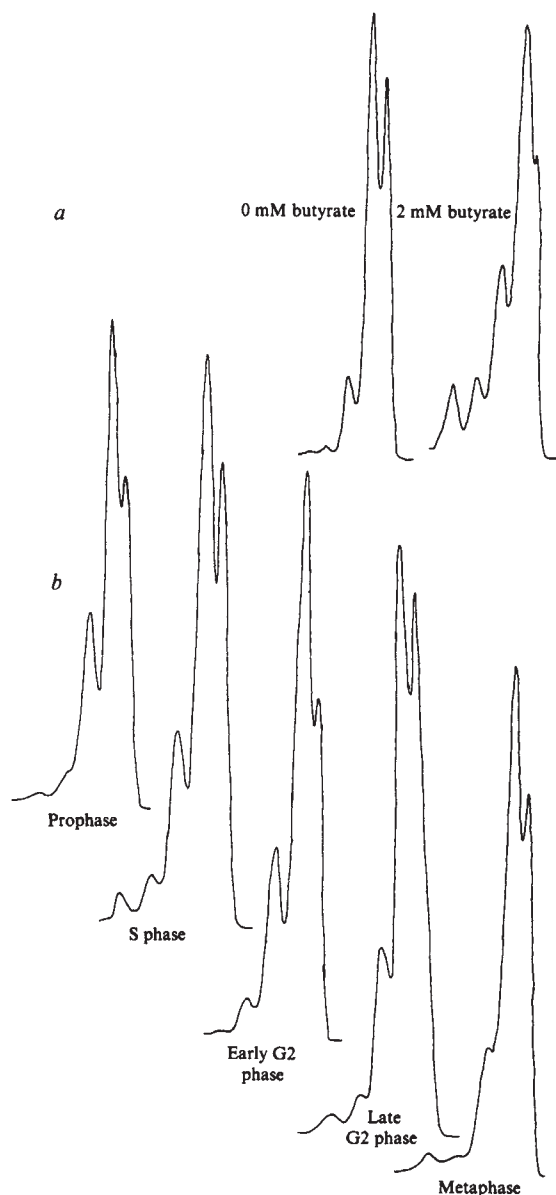


Fig. 2 Polyacrylamide gel electrophoresis of *Physarum* histone H4 in 16-cm long gels. Each profile shows five bands corresponding to 4, 3, 2, 1 or 0 acetates per molecule, reading from left to right in the direction of migration. *a*, H4 from microplasmidia grown in normal medium (left) or in medium containing 2 mM butyrate (right). *b*, H4 from macroplasmidia collected at the following stages of the mitotic cycle (from left to right): mitosis (M2 - 15 min), S phase (M2 + 1.75 h); early G2 phase (M2 + 5 h); late G2 phase (M2 + 7.5 h); and mitosis (M3). Each scan shows the H4 region of a 16-cm long tube gel electrophoresed in acetic acid plus urea³⁵. The gels were stained with amido black, destained by diffusion and scanned on a Joyce-Loebl gel scanner. M2 and M3 are the second and third mitosis after fusion, respectively. The cycle time was 11 h.

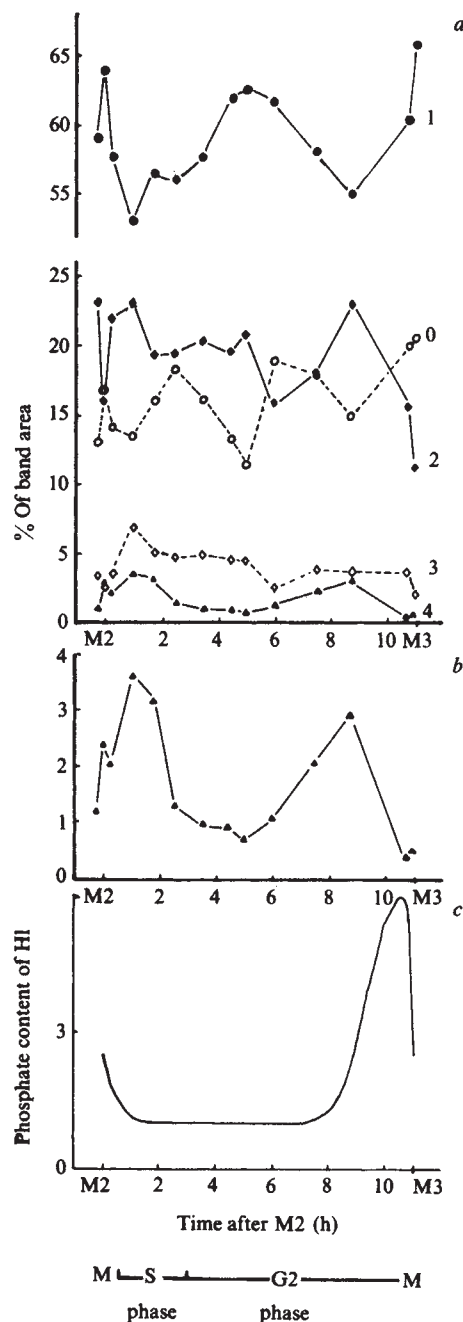


Fig. 3 Acetylated H4 through the cell cycle. *a*, ○—○ Percentage of total H4 in the non-acetylated form; ●—● percentage of total H4 in the mono-acetylated form, Ac₁H4; ◆—◆ percentage of total H4 in the di-acetylated form, Ac₂H4; ◇—◇ percentage of total H4 in the tri-acetylated form, Ac₃H4; ▲—▲ percentage of total H4 in the tetra-acetylated form, Ac₄H4. *b*, Percentage of Ac₄H4, shown on a larger scale. *c*, Relative phosphate content of *Physarum* histone H1 through the cell cycle⁵.

trypsin digestion²³ without a major effect on the integrity of the core particle. Consequently, acetylation of the N-terminal region might be expected to have very little effect on isolated single nucleosomes and preliminary structural studies of acetylated nucleosomes isolated from butyrate-treated HeLa S3 cells support this prediction^{24,25}. In the bulk of chromatin, which is transcriptionally inactive, nucleosomes are packed into a higher order structure—the 34-nm coil of Fig. 4 (refs 26–28). It is a reasonable assumption that inter-nucleosomal interactions of the basic N-terminal regions of core histones are involved in generating the 34-nm coil. In this case, acetylation, particularly multiple acetylation of the basic N-terminal, would destabilize the 34-nm coil, causing a transition to a more extended

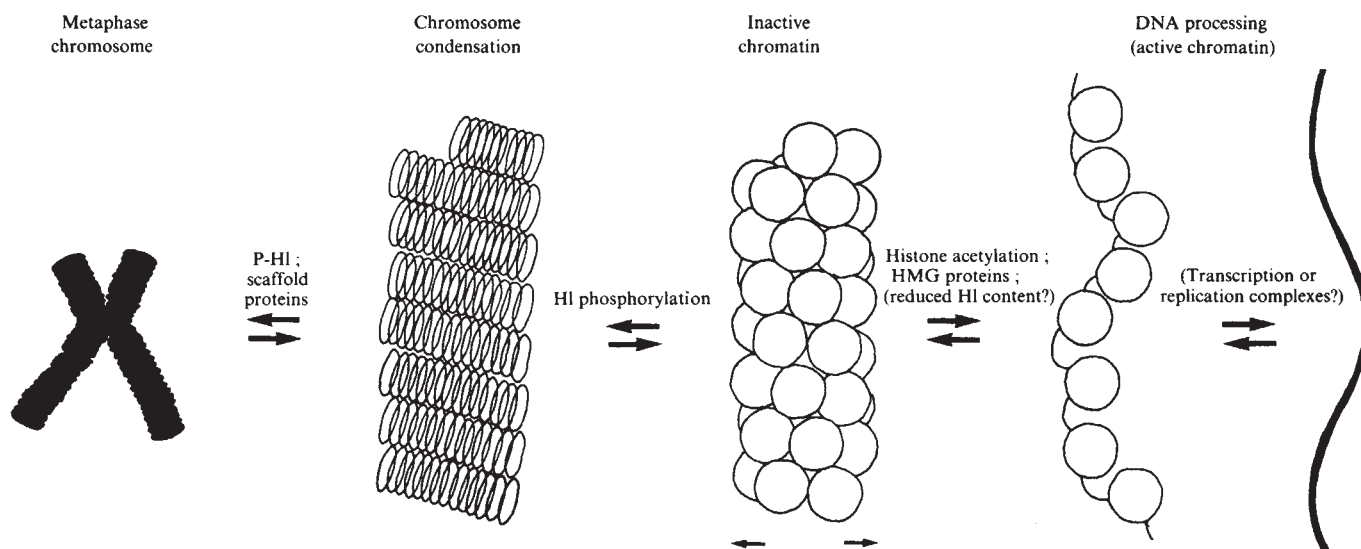


Fig. 4 Model representing the major structural transitions in chromatin. The different structural states are shown in diagrammatic form only.

conformation (Fig. 4). There is substantial evidence that transcriptionally active chromatin is in a more extended conformation than the bulk of chromatin (for example, see ref. 29) suggesting that the role of multiple H4 acetylation is to destabilize the inactive structure of chromatin (Fig. 4). Conversely, deacetylation of H4 would promote the formation of the inactive 34-nm coil and this is observed in mitosis (Fig. 3). Phosphorylation of H1 is probably involved in packing the 34-nm coils into the next order of structure (Fig. 4) and so it is inversely correlated with H4 acetylation. We anticipate one or two further orders of packing, in metaphase, leading to the metaphase chromosome and perhaps involving non-histone 'scaffold' proteins³⁰. Other non-histone proteins, particularly HMG proteins³¹⁻³⁴, are also involved in the active chromatin structure and our present understanding of the major structural transitions in chromatin is summarized in Fig. 4.

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- Dixon, G. H. *et al. Ciba Fdn Symp.* **28**, 229-258 (1975).
- Allfrey, V. G. in *Chromatin and Chromosome Structure* (eds Li, H. J. & Eckhardt, R. A.) 167-191 (Academic, New York, 1977).
- Mohberg, J. & Rusch, H. P. *Archs Biochem. Biophys.* **134**, 577-585 (1969).
- Corbett, S., Miller, S., Robinson, V. J., Matthews, H. R. & Bradbury, E. M. *Trans. biochem. Soc.* **5**, 943-946 (1977).
- Bradbury, E. M., Inglis, R. J., Matthews, H. R. & Sarnier, N. *Eur. J. Biochem.* **33**, 131-139 (1973).
- Riggs, M. G., Whittaker, R. G., Neumann, J. R. & Ingram, V. M. *Nature* **268**, 463-464 (1977).
- Ruiz-Carrillo, A., Waugh, L. J. & Allfrey, V. G. *Science* **190**, 117-128 (1975).
- Panyim, S. & Chalkley, R. *Biochemistry* **8**, 3972-3977 (1969).
- Jackson, V., Shires, A., Tanphaichitr & Chalkley, R. *J. molec. Biol.* **104**, 471-483 (1976).
- Braun, R., Mittermayer, C. & Rusch, H. P. *Biochim. biophys. Acta* **114**, 527-535 (1966).
- Mittermayer, C., Braun, R. & Rusch, H. P. *Biochim. biophys. Acta* **91**, 387-398 (1964).
- Grant, W. D. *Eur. J. Biochem.* **29**, 94-98 (1972).
- Davies, K. E. & Walker, I. O. *J. Cell Sci.* **26**, 267-279 (1977).
- Sauer, H. W. in *Cell Cycle Regulation* (eds Jeter, J. R. *et al.*) 149-156 (Academic, New York, 1978).
- Matthews, H. R. in *The Cell Cycle* (ed. Johns, P.) (Cambridge University Press, in the press).
- Molgaard, H. V., Matthews, H. R. & Bradbury, E. M. *Eur. J. Biochem.* **68**, 541-549 (1976).
- Vogt, V. & Braun, R. *J. molec. Biol.* **106**, 567-587 (1976).
- D'Anna, J. A., Tobey, R. A., Barham, S. S. & Gurley, L. R. *Biochem. biophys. Res. Commun.* **77**, 187-194 (1977).
- Lake, R. S. & Salzmann, N. P. *Biochemistry* **11**, 4817-4822 (1972).
- Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L. & Tobey, R. A. *Eur. J. Biochem.* **84**, 1-16 (1978).
- Matthews, H. R. in *Protein Phosphorylation in Regulation* (ed. Cohen, P.) (North-Holland, Amsterdam, 1978).
- Cary, P. D., Moss, T. & Bradbury, E. M. *Eur. J. Biochem.* **89**, 475-482 (1978).
- Whitlock, J. & Simpson, R. T. *J. biol. Chem.* **252**, 6516-6520 (1977).
- Vidali, G., Boffa, L. C., Bradbury, E. M. & Allfrey, V. G. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2239-2243 (1978).
- Mathis, D. J., Oudet, P., Wasylyk, B. & Chambon, P. *Nucleic Acids Res.* **5**, 3523-3547 (1978).
- Finch, J. T. & Klug, A. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1897-1901 (1976).
- Carpenter, B. G., Baldwin, J. P., Bradbury, E. M. & Ibel, K. *Nucleic Acids Res.* **3**, 1739-1746 (1976).
- Suau, P., Bradbury, E. M. & Baldwin, J. P. *Eur. J. Biochem.* **97**, 593-602 (1979).

- Johnson, E. M., Allfrey, V. G., Bradbury, E. M. & Matthews, H. R., *Proc. natn. Acad. Sci. U.S.A.* **75**, 1116-1120 (1978).
- Adolph, K. W., Cheng, S. M. & Laemmli, U. K. *Cell* **12**, 805-816 (1977).
- Goodwin, G. H. & Johns, E. W. *Eur. J. Biochem.* **40**, 215-220 (1973).
- Levy-W., B., Wong, N. C. W. & Dixon, G. H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2810-2814 (1977).
- Vidali, G., Boffa, L. & Allfrey, V. G. *Cell* **12**, 408-415 (1977).
- Weisbrod, S. & Weintraub, H. *Proc. natn. Acad. Sci. U.S.A.* **76**, 635-650 (1979).
- Laemmli, U. K. *Nature* **227**, 680-682 (1970).
- Borun, T. W., Ajiro, K., Zweidler, A., Dolby, T. W. & Stephens, R. E. *J. biol. Chem.* **252**, 173-180 (1977).

The putative mRNA for subunit II of human cytochrome c oxidase starts directly at the translation initiator codon

Deanna K. Ojala, Julio Montoya & Giuseppe Attardi

Division of Biology, California Institute of Technology, Pasadena, California 91125

The gene for subunit II of cytochrome c oxidase (COII) in human mitochondrial DNA (mtDNA) is immediately contiguous, on its 5'-end side, to a tRNA^{Asp} gene¹. Since all eukaryotic mRNAs so far analysed have been shown to have a noncoding stretch, which is presumably used for ribosome attachment^{2,3} on the 5'-side of the coding sequence, it was reasonable to ask whether, in the case of the human COII mRNA, the above function is performed by the tRNA^{Asp} sequence or a portion of it, or whether this mRNA lacks a 5' noncoding stretch. We have sequenced a 5'-end proximal segment of the putative COII mRNA from HeLa cells of about 30 nucleotides and have aligned it with the COII coding sequence in human mtDNA. Our results show that this RNA starts directly at the AUG initiator codon.

Figure 1 shows a region of the HpaII restriction map of HeLa cell mtDNA⁴ with the precise positions of the COII gene and of the adjacent putative tRNA genes, as determined from DNA sequencing analysis¹. Preliminary RNA mapping experiments using the Southern⁵ and Alwine *et al.*⁶ methods had shown that a single mtDNA heavy (H)-strand coded poly(A)-containing RNA (RNA species 16, in the classification of Amalric *et al.*⁷) maps in correspondence to the COII gene. This RNA, on account of its size (about 800 nucleotides), its abundance, its presence in polysomes and its relatively long half life, is probably the mRNA for subunit II. Recently, using the S₁ protection

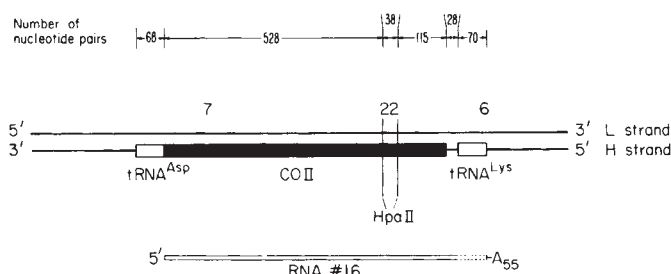


Fig. 1 Region of the *HpaII* restriction map of HeLa cell mtDNA⁴ showing the precise positions of the COII gene and of the adjacent tRNA genes. The positions of the genes were derived from the published DNA sequencing analysis¹. The mapping position of poly(A)-containing RNA 16 (ref. 10) is shown in the lower part of the figure; the dashed portion indicates the limits of uncertainty in this determination.

technique^{8,9}, it has been possible to locate the 5'-end of RNA 16 more precisely, and it has been placed at about 540 nucleotides from the *HpaII* site between fragments 7 and 22; in the same experiments, the 3'-end of the non-poly(A) segment of this RNA has been mapped in the region of the tRNA^{Lys} gene, with indications that it may overlap, in part, or possibly completely the tRNA^{Lys} coding sequence¹⁰ (Fig. 1).

To prepare RNA 16 of high purity and in adequate amounts for sequencing analysis, the micrococcal nuclease procedure for purification of mitochondrial RNA¹¹ was scaled up as described in the legend for Fig. 2. Lane *a* in this figure shows the electrophoretic pattern in a CH₃HgOH-agarose slab gel, after ethidium bromide staining, of the oligo(dT)-bound fraction of mitochondrial RNA derived from about 2.5×10^9 HeLa cells. The typical pattern of the mtDNA-coded poly(A)-containing RNA species previously described in HeLa cells⁷ is clearly recognizable, with the exception of the species known to be relatively short-lived and present in very small amounts (RNA species 1-4 and 6)^{12,13}. RNA species 16 was eluted from the gel track shown in Fig. 2*a* and an identical track, treated with RNase-free DNase (to eliminate any contaminating DNA fragments), and re-run on a gel in the same conditions. A sharp band was observed at the expected position (Fig. 2*b*) and the

RNA in this band was eluted. Material from about 8×10^9 cells, purified as described above, was labelled at its 5'-end with [γ -³²P]ATP and polynucleotide kinase after dephosphorylation with bacterial alkaline phosphatase. The labelled RNA was run on a CH₃HgOH-agarose gel, a band corresponding to intact RNA 16 was observed (Fig. 2*c*) and the RNA in this band was eluted for the sequencing reactions. Mitochondrial 12S rRNA, to be used as a control for the *in vitro* labelling and sequencing technology used in the present work, was purified from the oligo(dT)-unbound RNA fraction of micrococcal-nuclease treated mitochondria and labelled *in vitro* at its 5'-end as described above.

To characterize the 5'-terminus of RNA 16, the *in vitro* 5'-end labelled RNA was subjected to a complete P1 digestion¹⁴ and fractionation on a PEI-cellulose TLC plate. As a control, 5'-end-labelled 12S rRNA was subjected to the same analysis. As shown in Table 1, almost 92% of the radioactivity in the 12S rRNA was found to be associated with pA, the remainder being distributed among the three other nucleotides, in perfect agreement with previous work¹¹. In RNA 16, the predominant 5'-terminal nucleotide (~84%) was pA. This result indicated the substantial purity of the RNA 16 used for sequencing in the present work.

Sequencing of the 5'-end proximal segment of RNA 16 and of 12S rRNA was performed by the methods of Donis-Keller *et al.*¹⁵ and Simoncsits *et al.*¹⁶, using base-specific enzymatic

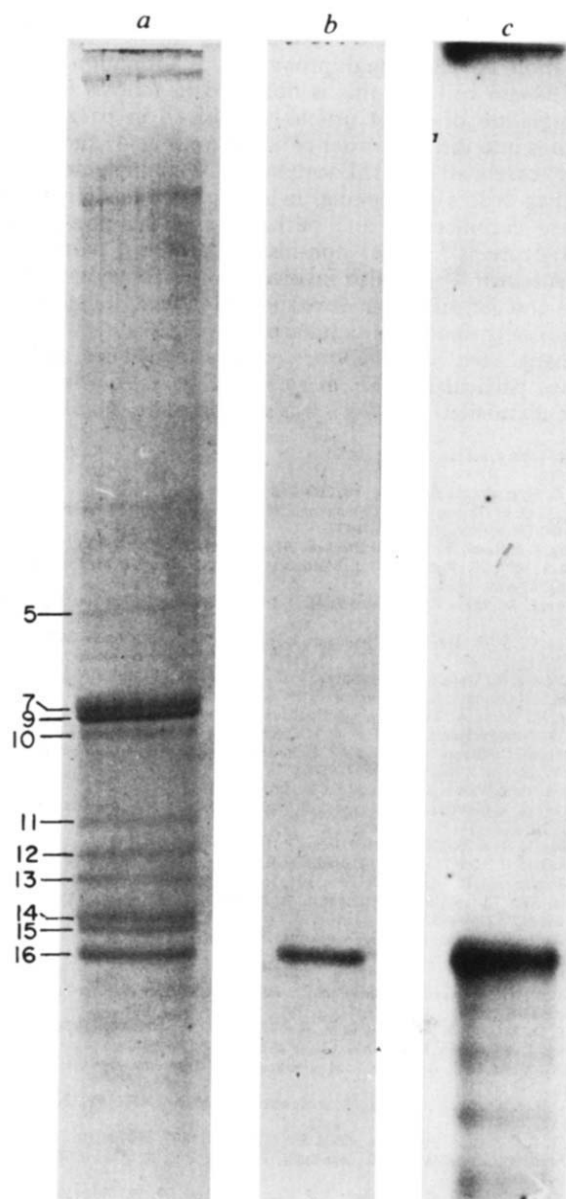
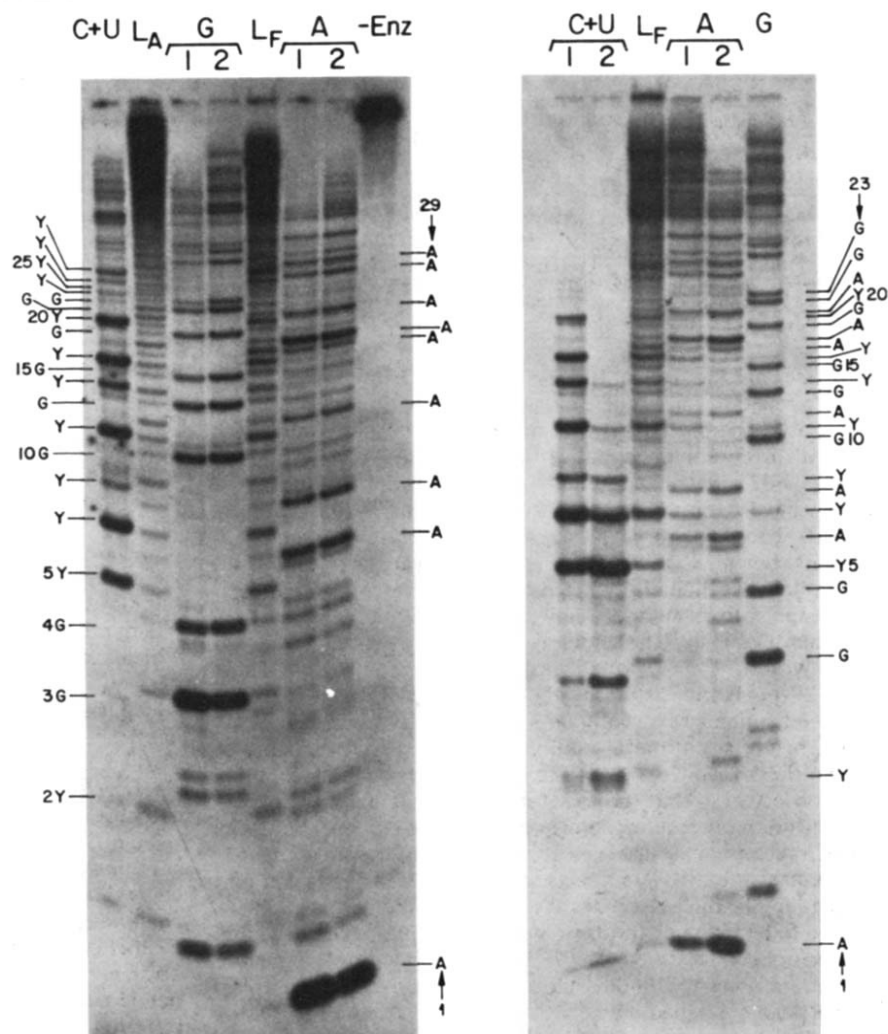


Fig. 2 Isolation and 5'-end labelling of mitochondrial poly(A)-containing RNA 16. *a*, The mitochondrial fraction from 5×10^9 HeLa cells (~30 ml packed cells) was re-suspended in 0.25 M sucrose, 0.01 M Tris-HCl, pH 8.0 (25 °C), 0.001 M CaCl₂ (1 ml per 1.5×10^8 cells), and treated with 100 units ml⁻¹ of micrococcal nuclease (Millipore) at 2 °C for 30 min. EGTA was then added to 2.5 mM and, after 5 min, the suspension was diluted with 0.25 M sucrose, 0.01 M Tris-HCl, pH 6.7, 0.01 M EDTA (10 ml per 1.5×10^8 cells), and the mitochondria were pelleted by centrifugation at 10,000 r.p.m. in a SS-34 rotor. The pellet was re-suspended in 0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.001 M EDTA (1 ml per 1.5×10^8 cells), incubated for 5 min at room temperature with 100 µg pronase per ml, then lysed with 1% SDS and incubated for an additional 30 min. RNA was extracted by phenol/chloroform/isoamyl alcohol (25:25:1) as previously described⁷, recovered by ethanol precipitation, denatured by heating at 63 °C for 4 min in 0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA and passed through an oligo(dT)-cellulose column. The bound fraction was eluted, heat-denatured again as described above and re-run through the oligo(dT)-cellulose column. The final bound fraction was collected by ethanol precipitation and run through two tracks of a CH₃HgOH-agarose (1.4%) slab gel⁷. *b*, The band in *a* which corresponds to poly(A)-containing RNA 16 was identified by ethidium bromide staining and eluted as previously described¹¹. After ethanol precipitation, the RNA was dissolved in 250 µl 0.05 M Tris-HCl, pH 6.7, 0.0025 M MgCl₂, 0.025 M KCl, incubated with 20 µg ml⁻¹ RNase-free DNase (Boehringer Mannheim), pronase-SDS-phenol extracted and re-run through a CH₃HgOH-agarose slab gel. *c*, The RNA 16 band in *b* was eluted, ethanol-precipitated, dissolved in 100 µl of 0.01 M Tris-HCl, pH 8.0, and incubated at 65 °C for 15 min with 100 units of bacterial alkaline phosphatase (BRL) per µg RNA (an amount of about 0.17 µg RNA 16 was roughly estimated to be recovered from $\sim 5 \times 10^9$ cells on the basis of its ethidium bromide staining, as compared to that of a known amount of 12S rRNA). Nitrilotriacetic acid was added to 10 mM and, after 15 min at room temperature, 2 µg of closed-circular pBR322 DNA were added as a carrier, and the nucleic acids were extracted with phenol/chloroform/isoamyl alcohol, ethanol-precipitated and dissolved in 15 µl kinase buffer (0.05 M Tris-HCl, pH 8.0, 0.01 M MgCl₂, 0.005 M dithiothreitol DTT) containing 0.5 nmol of [γ -³²P]ATP (8,000 Ci mmol⁻¹). After addition of 2 units of polynucleotide kinase (Boehringer Mannheim), the reaction was carried out at 37 °C for 15 min and then stopped by addition of 1.0 M ammonium acetate. Carrier tRNA was then added and the mixture ethanol-precipitated and run on a CH₃HgOH-agarose (1.4%) slab gel.

Fig. 3 RNA sequencing of mitochondrial poly(A)-containing RNA 16. Autoradiograms of 25% sequencing gels (showing nucleotides 1–29) of partial enzymatic digest of 5'-end labelled RNA 16. Shown at the top of the gel are the cleavage specificities: RNase U2 (A), RNase T1 (G), RNase A (C+U), alkali ladder (L_A), formamide ladder (L_F). The reaction conditions described by Donis-Keller *et al.*¹⁵ were used. The amounts of enzymes used (per ~30 ng RNA 16 and 3 µg yeast tRNA carrier) were, in the left panel: A 0.25 ng; T1 (1, 2), 0.10, 0.05 units; U2 (1, 2), 1.0, 0.5 units; in the right panel: A (1, 2), 0.50, 0.75 ng; T1, 0.02 units; U2, 0.15, 0.5 units. The alkali and hot formamide degradations for the formation of the ladder were performed as described by Donis-Keller *et al.*¹⁵ and by Simoncsits *et al.*¹⁶, respectively. Electrophoresis was carried out on thin (0.5 mm) 25% polyacrylamide (30:1 bisacrylamide)/7 M urea gels in Tris-borate-EDTA buffer at 1,000 V for 6 h.



cleavage of the RNA followed by electrophoresis on polyacrylamide/urea gels. The RNases T1 (Sigma) (G-specific), A (Sigma) (C+U-specific) and U2 (Sankyo) (A-specific) were used for this analysis. Since the purpose of this work was the alignment of the RNA sequence with the known DNA sequence, the precise identification of the pyrimidines was considered unnecessary.

The 12S rRNA gel patterns (not shown) yielded a 5'-end-proximal sequence identical to that previously shown for this RNA species¹¹. The gel patterns for RNA 16 (Fig. 3) gave a 5'-end proximal sequence which could be read for 29 nucleotides (Fig. 3). The band corresponding to the second nucleotide (a pyrimidine) was very faint in the RNA sample digested with

0.25 ng RNase A (Fig. 3, left panel), but was clearly visible in the sample digested with higher enzyme levels (Fig. 3, right panel). There were a few extra bands in the region corresponding to small oligonucleotides after RNase U2 or T1 digestion (and also after treatment of the sample with the higher levels of RNase A). These bands were in most cases less strong than the *bona fide* specific RNase cleavage products; because of this and because they did not correspond in migration to bands visible in the ladder or corresponded to obviously abnormal ladder steps (like the fairly strong band corresponding to a pyrimidine in the second lane of the right panel), they could in general be recognized as spurious bands. The nature of these extra bands is not clear; they may reflect the presence of contaminating oligo-

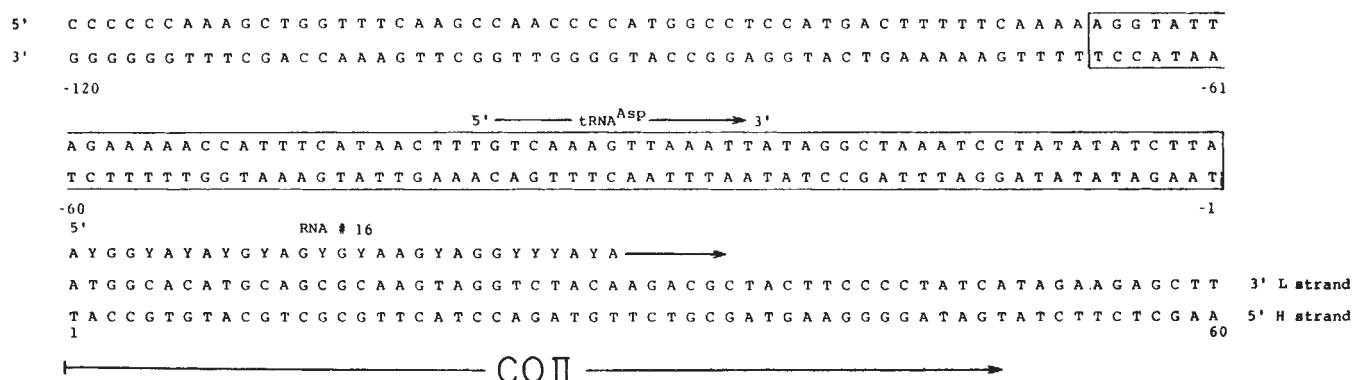


Fig. 4 Alignment of the nucleotide sequence of the 5'-end region of poly(A)-containing RNA 16 with the DNA sequence of the COII gene. The RNA sequence was derived from Fig. 3. The sequence of the 5'-end-proximal region of the COII gene and of the adjacent DNA segment on its 5'-side is reproduced from Barrell *et al.*¹. Y, Pyrimidine.

Table 1 5'-terminal nucleotide analysis of *in vitro* labelled mitochondrial poly(A)-containing RNA 16 and 12S rRNA

RNA species	Percentage of total ³² P radioactivity*			
	AMP	CMP	GMP	UMP
12S rRNA	91.8	1.9	2.0	4.2
Poly(A)-containing RNA 16	83.6	1.3	8.2	7.0

The 5'-terminal nucleotide was determined by exhaustive digestion of the *in vitro* labelled RNAs with nuclease P1 (ref. 14; Calbiochem) and fractionation of the products on PEI-cellulose TLC plates, as previously described¹¹.

* The values refer to the radioactivity which migrated from the origin. The percentage of the input radioactivity recovered from the origin was, respectively, 1.0 and 1.6% for 12S rRNA and RNA 16.

nucleotides in the RNA 16 preparation, during the 5'-end labelling reaction, or may be due to the formation, during the enzymatic or chemical degradation of the RNA, of oligonucleotides with different phosphate end-groups (2',3'-cyclic phosphate, 2' and 3' phosphate). Similar bands migrating in abnormal position have been observed, previously¹¹ and in the present work, in the sequencing gel patterns for 12S rRNA. The reading of the first few nucleotides of the sequence near the 5'-end was made somewhat difficult by the presence of these extra bands; beyond the fifth nucleotide, however, the sequence could be read unambiguously. The sequence obtained is shown in Fig. 4, aligned with the DNA sequence of the COII gene region. The entire 29-nucleotide stretch of RNA 16 which has been sequenced appears to be colinear with the DNA sequence. The striking result is that the 5'-end of the RNA corresponds precisely to the first nucleotide of the COII coding sequence.

The observation that the COII mRNA starts directly at the initiator codon raises interesting questions about the mechanism by which the mitochondrial ribosomes attach to this messenger. In all eukaryotic mRNAs analysed so far there is a stretch of variable length which precedes the initiator codon and which is assumed to be involved in ribosome attachment^{2,3}. Similarly, in *Escherichia coli* mRNAs there is a 5'-noncoding segment containing a ribosome binding site^{17,18}. A single exception to this rule has been described, namely that of the λ repressor mRNA involved in repressor maintenance¹⁹. In this case, it was argued that the lack of a strong ribosome binding site could account for the low efficiency of translation of this mRNA. In the case of the mitochondria from human cells (and probably from other animal cells) it is reasonable to think that the special features of their ribosomes make them suitable for binding directly to the initiator codon. It will be interesting to see whether the lack of a 5'-noncoding stretch is a general feature of mitochondrial mRNAs in HeLa cells. Preliminary observations indicate that another mitochondrial poly(A)-containing RNA in these cells, RNA 15, starts with an AUG, which may be the initiator codon for the polypeptide coded by this RNA (unpublished observations).

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1. Barrell, B. G., Bankier, A. T. & Drouin, J. *Nature* **282**, 189-194 (1979).
2. Hagenbüchle, O., Santer, M., Steitz, J. A. & Mans, R. J. *Cell* **13**, 551-563 (1978).
3. Kozak, M. *Cell* **15**, 1109-1123 (1978).
4. Ojala, D. & Attardi, G. *Plasmid* **1**, 78-105 (1977).
5. Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
6. Alwine, J. C., Kemp, D. J. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5350-5354 (1977).
7. Amalric, F., Merkel, C., Gelfand, R. & Attardi, G. *J. molec. Biol.* **118**, 1-25 (1978).
8. Berk, A. J. & Sharp, P. A. *Cell* **12**, 721-732 (1977).
9. Berk, A. J. & Sharp, P. A. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1274-1278 (1978).
10. Ojala, D. K., Merkel, C., Gelfand, R. & Attardi, G. (in preparation).
11. Crews, S. & Attardi, G. *Cell* **19**, 775-784 (1980).
12. Attardi, G. *et al.* *ICN-UCLA Symp. molec. cell. Biol.* **15**, 443-469 (1979).
13. Gelfand, R. thesis, California Institute of Technology (1980).
14. Fujimoto, M., Kuninaka, A. & Yoshino, H. *Agr. Biol. Chem.* **38**, 1555-1561.
15. Donis-Keller, H., Maxam, A. M. & Gilbert, W. *Nucleic Acids Res.* **4**, 2527-2538 (1977).
16. Simoncsits, A., Brownlee, G. G., Brown, R. S., Rubin, J. R. & Guillely, H. *Nature* **269**, 833-836 (1977).
17. Shine, J. & Dalgarno, L. *Biochem. J.* **141**, 609-615 (1974).
18. Steitz, J. A. & Jakes, K. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4734-4738 (1975).
19. Ptashne, M. *et al.* *Science* **194**, 156-161 (1976).

Structural and functional diversity in 4- α -helical proteins

Patricia C. Weber & F. R. Salemme

Department of Biochemistry, New Chemistry Building, University of Arizona, Tucson, Arizona 85721

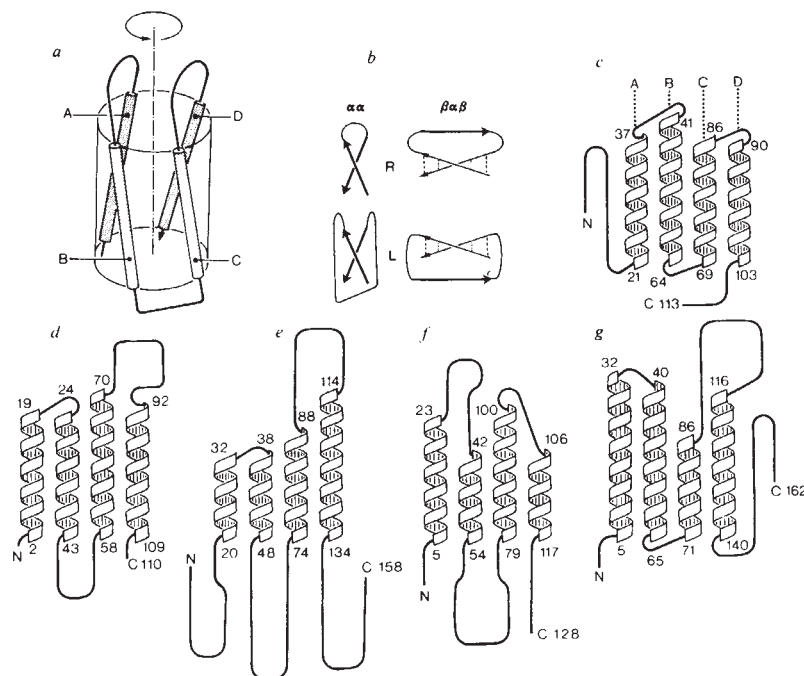
Protein crystallographic studies show that many structural arrangements appear as common features among proteins which are otherwise unrelated in sequence or function. One of the more recently recognized recurring protein structural motifs is a nearly parallel arrangement of four α -helices to form a sequentially connected left-twisted bundle¹. We describe here the geometrical properties of these structures and suggest how they relate to the functional and aggregate properties of these molecules.

Currently known proteins which incorporate a 4- α -helical bundle as their dominant structural feature include (1) the haemerythrin subunit and related monomer myohaemerythrin, which are non-haem iron-containing oxygen transport proteins derived from marine worms²⁻⁴, (2) the apoferritin monomer, which associates to form a hollow, 24-subunit cube-octahedral aggregate functioning in iron storage⁵, (3) tobacco mosaic virus (TMV) coat protein^{6,7}, (4) the monomeric haem protein, *Escherichia coli* cytochrome *b*₅₆₂ (ref. 8), and (5) the dimeric cytochrome *c'* from *Rhodospirillum rubrum*⁹.

Figure 1 schematically compares the known 4- α -helical subunit structures in cylindrical projection. Although the molecules clearly differ in their α -helix, connecting loop, and overall chain lengths, they share a similar overall folding plan in that each is composed of four sequentially joined α -helices (A-D) arranged in a roughly fourfold symmetrical bundle having a net left-handed twist. As shown in recent treatises on helix packing^{10,11}, individual α -helices can be described, to a first approximation, as twisted parallelepipeds having a square cross-section. It is consequently obvious that the observed arrangement of four nearly-parallel α -helices to form an array having a square cross-section minimizes the accessible surface area of the structure as a whole¹². Additionally, if it is assumed that these molecules fold from an intermediate state having preformed α -helices^{10,13}, it can be seen from inspection of Fig. 1 that all sequential helix pairs and intervening connecting loops have a net right-handed superhelical character. This is a well known chiral effect observed in several other protein structural domains¹⁴⁻²³ and is presumed to arise through some intrinsic property of extended polypeptide chains that confers stability on conformations having overall right-handed supercoil characteristics^{24,25}. In the present case, these right-handed supercoiled connections provide the most efficient connectivity pattern for the bundle, since alternative left-supercoiled connections would necessitate the formation of a much larger loop passing around the entire molecule (Fig. 1).

Analysis of the available coordinate²⁶ or literature data²⁷ shows the helix packing in these molecules to be quite intimate, the mean adjacent interhelix axis distance of closest approach being 9.6 Å, with a standard deviation of 1.4 Å. Interhelix angles are remarkably similar, the mean interhelical angles for adjacent and diagonally related helix pairs being $162.0 \pm 5.8^\circ$ and $-158.2 \pm 7.0^\circ$ (mean $\pm$ s.d.), respectively¹⁰. The near constancy of the adjacent pair interhelix axis angle at $\sim 18^\circ$ ($180^\circ - 162^\circ$) reflects the fact that the individual helices can only make intimate and extended packing arrangements when their axes are relatively tilted at an angle equal to twice the α -helix pitch angle ($\sim 9.0^\circ$; ref. 28). Although a detailed description of all the 4-fold symmetric, sterically acceptable 4- α -helical arrangements lies outside the scope of this communication, it can readily be demonstrated that in fact there exists a variety of

Fig. 1 Schematic representations of the 4- α -helical protein structures. *a*, Schematic representation of helical packing; *b*, schematic representation of chiral connectivity; *c*–*g*, examples of proteins showing these characteristics: *c*, haemerythrin and myohaemerythrin; *d*, cytochrome b_{562} ; *e*, TMV protein; *f*, cytochrome c' ; *g*, apoferritin. Note that the connectivity pattern shown here and in Fig. 3 of the apoferritin monomer is one of two possibilities (the alternative being left-handed⁵) either of which appears equally consistent with the current electron density map interpretation (P. M. Harrison, personal communication). Each molecule is illustrated as a cylindrical projection of a common arrangement of four α -helices which are sequentially connected to form a bundle having an overall left-handed twist about the approximate 4-fold bundle axis of symmetry. This arrangement results in a right-handed (R) supercoiled connectivity pattern between adjacent helices, a feature previously observed in several other protein structural domains^{14–23}. In *b*, for example, we compare right- and left-hand connected helix pairs with analogous β - α - β domains.

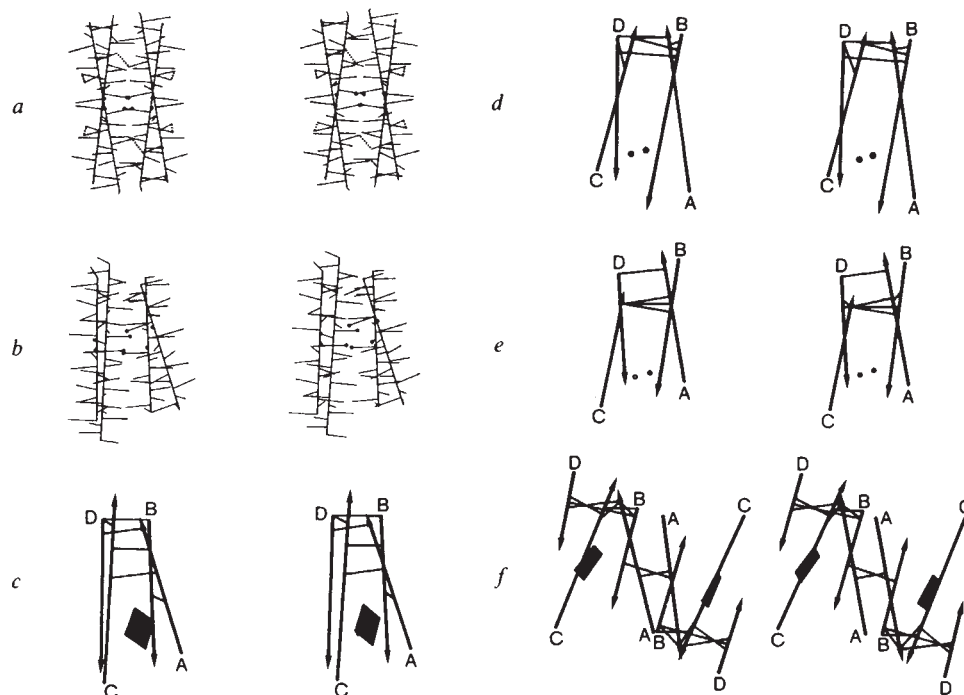


related packing arrangements which are both consistent with the observed geometry and have overall symmetric interactions. Further, as can be shown by an appropriate helix-net representation²⁸, the interhelix angle of 18° is a value which is uniquely associated with the attainment of regular periodic interactions between all helix pairs in a 4- α -helical bundle. In Fig. 2*a*, we show a packing model²⁸ of a representative symmetric arrangement which illustrates these points, and a similar representation of the actual packing interactions in cytochrome b_{562} is shown in Fig. 2*b*. These structures are clearly related, and so the previously reported occurrences of high internal sym-

metry in 4- α -helical structures^{27,29} would seem to reflect the symmetrical packing requirements for helices in these bundles.

Because the α -helices in the 4- α -helical bundle structures are straight, they must necessarily diverge from their point of closest interhelix axis approach. Figure 2*c*–*f* illustrates this behaviour for several 4- α -helical proteins, and demonstrates both that the helical bundles of these structures have been truncated near their points of closest interhelix axis approach, and that the spatial divergence of the helices, which results as a consequence of the helix packing requirements, serves to create the internal binding pocket for the incorporated prosthetic groups.

Fig. 2 Packing geometry in 4- α -helical proteins. *a*, A 'broomstick' packing model²⁸ in which each helix is represented as a helix axis line with 5.6 Å radial vectors¹⁰ passing through $C\beta$. All adjacent helix pairs have interhelix axis angles of 18° and interaxis close-approach distances of 9.6 Å, so that the structure as a whole has the average geometrical properties of the observed structures. The structure shown has nearly equivalent local packing interactions (for example, dotted connection) which approximately repeat in the direction of the long axis of the bundle. (We note here that the interactions between the residues of straight α -helices can, at best, be only quasi-equivalent along the bundle axis. This situation differs from that observed in superhelical fibrous structures²⁸.) The arrangement as a whole has 222 point group symmetry, as a consequence of which each face of the bundle presents a packing surface with a diad axis of symmetry. The structure shown is only one of many possible symmetrically related arrangements which share a common overall geometry. *b* Shows a broomstick representation of the helix packing interactions in cytochrome b_{562} (ref. 8). The packing interactions in this molecule are not as regular as the model in *a*, because the residues of the real protein have different packing volumes, and the helical bundle comprising this molecule has been truncated near the region of closest interhelix approach (see *c*), with a concomitant reduction in apparent packing symmetry. Nevertheless, comparison of *a* and *b* clearly shows the helix packing in these structures to be related. *c*–*f* Show helix axis representations (including close approach interhelix vectors) for several molecules. As shown, all are truncated versions of a generalized 4- α -helical bundle model, which incorporate prosthetic groups in the internal cavity created by the spatial divergence of the individual helices away from their points of closest approach. An arrow indicates the N to C polypeptide chain sense in each helix. *c*, Cytochrome b_{562} (ref. 8). The position of the noncovalently bound haem group is indicated by a solid square. *d*, Myohaemerythrin². Solid circles indicate the location of the bound iron atoms. *e*, Haemerythrin⁴. Iron positions are largely conjectural. *f*, Cytochrome c' dimer⁹ showing positions of the covalently bound haem prosthetic groups.



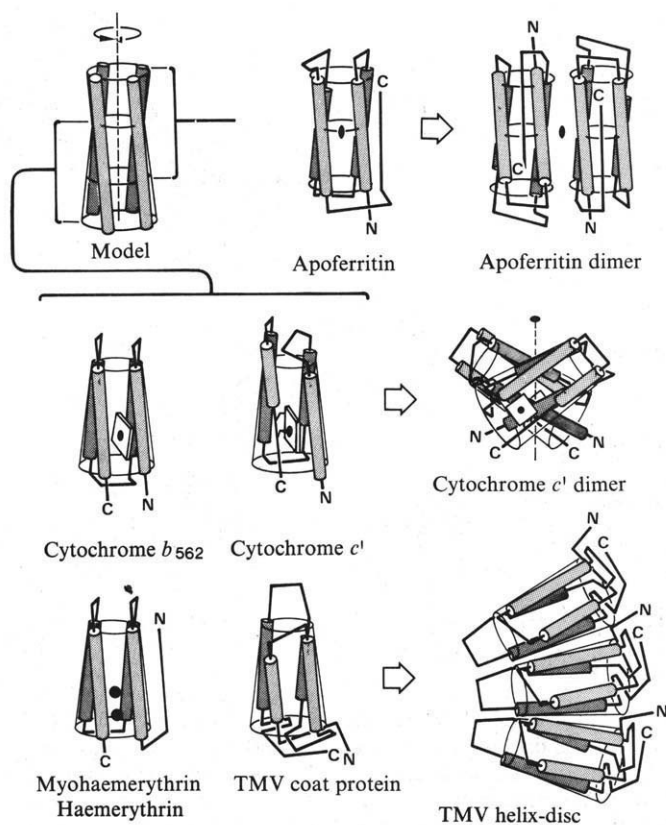


Fig. 3 Schematic representations of the 4- α -helical proteins illustrated as truncated variations of a generalized 4- α -helical packing model. Broad arrows indicate the relationship between the monomeric and aggregate structural properties of the respective molecules. Details are given in the text.

Figure 3 shows the known proteins of this class illustrated as truncated variations of a generalized 4- α -helical packing model, and summarizes some additional features of their structural and aggregate properties. For example, in addition to the molecules described in Fig. 2, TMV coat protein also clearly appears to be a truncated divergent bundle structure, which instead of incorporating a prosthetic group, has a β -sheet at its divergent end which serves to 'brace' the helices apart⁶. The apoferritin monomer, in contrast, incorporates neither of these features⁵ and so its helices pack more symmetrically with an equal extent of interhelical divergence at either end of the bundle. As a consequence, the most extensive pairwise monomer interaction in the cube-octahedral aggregate structure is a symmetrical dimer (Fig. 3) having interhelical interactions which are geometrically similar to those within each monomer⁵. As described in Fig. 2a, the symmetrical 4- α -helical packing arrangements which best satisfy the steric requirements of interhelical packing also generate diad axes of symmetry relating surface residues in adjacent helix pairs. As a result, the apoferritin subunits can pack to generate a dimeric structure whose symmetry is similar to that of the monomer.

The truncation of the divergent bundles of the remaining structures precludes the existence of diad symmetry axes on their faces, with a consequent reduction of their aggregate packing symmetry. In cytochrome *c'* for example, the monomers pack about a molecular diad axis which is independent of any symmetry elements of the monomer helix arrangements (Fig. 2).

TMV coat protein differs from the preceding molecules in that it forms aggregates having either 17- or 16.3-fold radial symmetry^{6,7} in which each monomer makes different sets of pairwise helical interactions with its neighbours. However, to a good approximation, the interhelical angle between diagonally related parallel helices of the truncated divergent bundle defines the apical angle of a pyramidal prism circumscribing the molecular subunit (Fig. 3). The average observed value for this angle

is 22° (180° - 158°, see above), which corresponds closely to the angle subtended by each subunit in both the TMV disk (360°/17 = 21.2°) and helix (360°/16.3 = 22.1°) structures.

To summarize, it has been shown here that the structural similarity among the 4- α -helical proteins can be attributed to what are basically physical requirements of helix packing and chiral connectivity. Consequently, it is not surprising that this structural arrangement should recur throughout the course of evolution, since it evidently possesses considerable scope for functional and superstructural diversity.

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- Argos, P., Rossmann, M. G. & Johnson, J. E. *Biochem. biophys. Res. Commun.* **75**, 83-86 (1977).
- Ward, K. B., Hendrickson, W. A. & Klippenstein, G. L. *Nature* **257**, 818-821 (1975).
- Hendrickson, W. A., Klippenstein, G. L. & Ward, K. B. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2160-2164 (1975).
- Stenkamp, R. E., Sieker, L. C., Jensen, L. H. & McQueen, J. E. Jr *Biochemistry* **17**, 2499-2504 (1978).
- Banyard, S. H., Stammers, D. K. & Harrison, P. M. *Nature* **271**, 282-284 (1978).
- Bloomer, A. C., Champness, J. N., Bricogne, G., Staden, R. & Klug, A. *Nature* **276**, 362-368 (1978).
- Stubbs, G., Warren, S. & Holmes, K. *Nature* **267**, 216-221 (1977).
- Mathews, F. S., Bethge, P. H. & Czerwinski, E. W. *J. biol. Chem.* **254**, 1699-1706 (1979).
- Weber, P. C. et al., *Nature* **286**, 302-304 (1980).
- Richmond, T. J. & Richards, F. M. *J. molec. Biol.* **119**, 537-555 (1978).
- Efimov, A. V. *J. molec. Biol.* **134**, 23-40 (1979).
- Richards, F. M. A. *Rev. Biophys. Bioengng* **6**, 151-175 (1977).
- Puitsyn, O. B. & Rashin, A. A. *Dokl. Acad. Nauk SSSR* **213**, 473-475.
- Rao, S. T. & Rossmann, M. G. *J. molec. Biol.* **76**, 241-256 (1973).
- Rossmann, M. G. & Liljas, A. *J. molec. Biol.* **85**, 177-181 (1974).
- Rossmann, M. G. & Argos, P. *J. molec. Biol.* **105**, 75-95 (1976).
- Richardson, J. S., Richardson, D. C., Thomas, K. A., Silverton, E. W. & Davies, D. R. *J. molec. Biol.* **102**, 221-235 (1976).
- Levitt, M. & Chothia, C. *Nature* **261**, 552-557 (1976).
- Richardson, J. S. *Nature* **268**, 495-500 (1977).
- Chothia, C. *J. molec. Biol.* **75**, 295-302 (1973).
- Sternberg, M. J. E. & Thornton, J. M. *J. molec. Biol.* **105**, 367-382 (1976).
- Richardson, J. S. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2619-2623 (1976).
- Sternberg, M. J. E. & Thornton, J. M. *J. molec. Biol.* **110**, 269-283 (1977).
- Weatherford, D. W. & Salemme, F. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 19-23 (1979).
- Ramachandran, G. N., Lakshminarayan, A. V. & Kolaskar, A. S. *Biochim. biophys. Acta* **303**, 8-13 (1973).
- Bernstein, F. C. et al. *J. molec. Biol.* **112**, 535-542 (1977).
- McLachlan, A. D., Bloomer, A. C. & Butler, P. J. G. *J. molec. Biol.* **136**, 203-224 (1980).
- Crick, F. H. C. *Acta crystallogr.* **6**, 689-697 (1953).
- Hendrickson, W. A. & Ward, K. B. *J. biol. Chem.* **252**, 3012-3018 (1977).

Do the chromosomes of the kiwi provide evidence for a monophyletic origin of the ratites?

Leobert E. M. de Boer

Biological Research Department, Royal Rotterdam Zoological and Botanical Gardens, Rotterdam, The Netherlands

The extensive literature on the origin of the ratites focuses mainly on three questions: are the ratites mono- or polyphyletic, did they evolve from flying ancestors, and are they primitive or advanced? Opinion tends to accept a common descent from flying ancestors for the large ratites (for a summary of ideas see ref. 1). They would have evolved on Gondwanaland some time in the Cretaceous and have become dispersed over the southern continents after its fragmentation^{2,3}. However, the position of the small New Zealand kiwis, in many respects the most peculiar of all birds, is still a matter for conjecture^{1,4}. The chromosome complements of the large ratites have been found to be remarkably uniform⁵⁻⁸. The chromosome set of the kiwi, described here, clearly links up with these, which may be recorded as another indication for monophyly of all ratites. It also indicates that we are dealing here with very ancient karyotypes.

The somatic chromosome complements of the five large ratites previously studied—the ostrich, emu, Australian cassowary and common and Darwin's rhea—proved to be almost identical^{5,8}. Minor differences only exist in the third and fifth chromosome pair. This result seems to support a monophyletic origin of this group, and indicates an extreme evolutionary conservatism of their karyotypic morphology, because geographical isolation of their ancestors on the various southern continents caused by the fragmentation of Gondwanaland took place probably 80–90 Myr ago^{2,4}. There have been no reports on the chromosomes of the kiwi. Thus, when one of the kiwis (*Apteryx australis*) at the Rotterdam Zoological Gardens recently died unexpectedly, blood and bone marrow were removed posthumously to obtain chromosome preparations (for lymphocyte culturing techniques see refs 9, 10). Possibly as a result of the infectious disease which had killed this specimen (which on postmortem observation was found to be a female), the cultures did not yield optimal results. Thus, chromosome banding studies could not be undertaken, with the exception of C-banding. Nevertheless, the available material permitted a good comparison with the data from the large ratites.

The chromosome set of the kiwi is composed of 80 chromosomes, with 6 pairs of macrochromosomes clearly distinguishable from 34 pairs of microchromosomes (Fig. 1). With the exception of the first and second pairs, all elements have a terminal or nearly terminal centromeric position. The chromosomes of the sixth pair show a weak secondary constriction in the subcentromeric region. The absence of a clearly heteromorphic sex chromosome pair is characteristic in the female specimen studied. In all other birds, with the exception of the ratites, the female is the heterogametic sex in that female individuals possess one Z and one W chromosome (which is generally much smaller) in the sex chromosome pair, whereas males possess two identical Z chromosomes. In the kiwi the fourth chromosome pair is only slightly heteromorphic, one member being consistently slightly smaller than the other. It is therefore possible that these elements represent the sex chromosomes. However, C-banding, a technique used specifically for staining the avian W chromosome, did not indicate the presence of a C-positive element among the macrochromosomes.

This exceptional situation, the absence of a clearly heteromorphic sex chromosome pair and the presence of a slight length difference in the fourth pair of chromosomes, has been found in the ostrich, cassowary, emu and rhea^{5–7}. Only in Darwin's rhea have Benirschke *et al.*⁸ claimed to have found a normal sex chromosome pair with a large Z and small W chromosome. In all male ratites studied, all chromosome pairs were perfectly homomorphic.

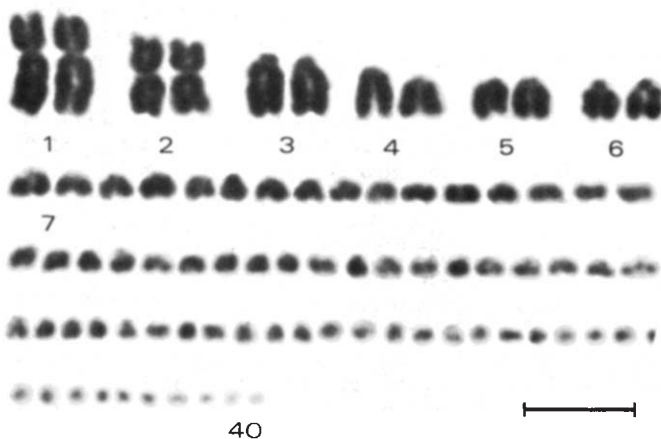


Fig. 1 Representative karyogramme of a female kiwi, *Apteryx australis*, obtained from lymphocyte culture. Note slight difference in length between the members of pair 4. The microchromosomes (pairs 7–40) have not been matched in pairs. Scale bar, 5 μ m of absolute length.



Fig. 2 Karyogramme of a female emu, *Dromaius novaehollandiae* (specifications as in Fig. 1).

The structure of the remainder of the kiwi karyotype also closely resembles those of the large ratites. In fact, the kiwi chromosomes are indistinguishable from those of the emu (Fig. 2) and cassowary. The karyotype of the ostrich differs only in the possible absence of the secondary constriction in the sixth pair and the somewhat smaller short arm in the third pair. The rhea karyotypes differ only by the submedian position of the centromere in the elements of the fifth pair.

Obviously, these gross morphological similarities need to be confirmed by detailed comparisons of chromosome banding patterns based on material from further specimens. Nevertheless, it is obvious that with its karyological characteristics the kiwi perfectly falls within the ratite group. As convergent evolution of karyotype morphology seems improbable, these findings strongly suggest a monophyletic origin of all ratites, including kiwis (and their relatives, the extinct New Zealand moas). However, one problem of this conclusion is that in cladistic¹¹ terms monophyly can only be concluded on the basis of shared derived characters, not on primitive ones. At present it is impossible to determine whether ratite karyotypic structure is primitive or not. Comparable karyotypes possibly only occur in the unrelated Anseriformes⁷, but the sex determining system of the ratites is unique among birds. Further investigation should determine whether this system is primitive, as might be suggested by the fact that clearly heteromorphic sex chromosome pairs are absent in many reptilian groups¹². Neither can the question of the position of the tinamous, often cited as possibly resembling the flying ancestors of the ratites, be answered from a karyological point of view, because the only report on tinamou chromosomes¹³ does not include adequate descriptions or illustrations.

Monophyly or polyphyly apart, there is no doubt of the ancient character of the ratite karyotypic structure. Cracraft⁴ places the ostrich–rhea bifurcation, which is generally believed to be the most recent one between the major ratite groups, as far back as 80–90 Myr ago. In the case of monophyly, the branching off of the kiwi and moa ancestors certainly would have been the earliest in ratite history. This would mean that the ratite karyotypes, especially those of kiwi, emu and cassowary, because of their uniformity, have remained practically unchanged during a period of possibly well over 100 Myr. In the case of polyphyly this would have been as long or even longer.

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1. Sibley, C. G. & Ahlquist, J. E. *Bull. Peabody Mus. nat. Hist.* **39**, 1–276 (1972).
2. Fooden, J. *Science* **175**, 894–898 (1972).
3. Cracraft, J. *J. zool. Res.* **169**, 455–545 (1973).
4. Cracraft, J. *Ibis* **116**, 494–521 (1974).
5. Sasaki, M., Ikeuchi, T. & Makino, S. *Experientia* **24**, 1292–1293 (1968).
6. Takagi, N., Itoh, M. & Sasaki, M. *Chromosoma* **36**, 281–291 (1972).
7. Takagi, N. & Sasaki, M. *Chromosoma* **46**, 91–120 (1974).
8. Benirschke, R. J., Sekulovich, R. E. & Risser, A. C. *Chromosome Inf. Serv.* **21**, 13–14 (1976).
9. Boer, L. E. M. de. *Genen Phaenen* **17**, 1–115 (1974).
10. Boer, L. E. M. de. *Genetica* **46**, 77–113 (1976).
11. Hennig, W. *Phylogenetic Systematics* (Urbana University Press, 1966).
12. Gorman, G. C. in *Cytotaxonomy and Vertebrate Evolution* (eds Chiarelli, A. B. & Capanna, E.) (Academic, London, 1973).
13. Lucca, E. J. de. *Rev. Bras. Pesquisas méd. Biol.* **7**, 253–263 (1974).

MATTERS ARISING

Cometary collisions on the Moon and Mercury

It has been proposed that unusual bright and dark swirl-like markings on the Moon and Mercury may be strongly magnetized residues of recent (<100 Myr) collisions of the latter bodies with one or more cometary comas¹. The photogeological investigations which led to this hypothesis were stimulated, at least in part, by emerging evidence that relatively high-amplitude magnetic anomalies detected with instruments on-board the Apollo subsatellites are concentrated in areas where swirls are found^{2,3}. Although we agree that many of the swirls are probably relatively strongly magnetized deposits of impact-generated material, we consider that meteoroid impacts occurring over a span of time comparable to the age of the Solar System can explain their existence at least as well as can recent comet impacts. Our reasons are given below.

First, the inferred sources of medium-amplitude magnetic anomalies on the central near side^{2,3} and on the far side⁴ are basin ejecta materials. This result is particularly well established on the near side, where anomalies are correlated with exposed segments of primary Imbrium basin ejecta (the Fra Mauro Formation) surrounded by poorly magnetized mare basalt. Both returned sample studies and surface magnetic field investigations support the contention that ejecta materials produced during meteoroid impacts are major sources of magnetic anomalies detected from orbit^{5,6}. Therefore, a special explanation for the high-amplitude anomalies involving "high-velocity imprinting of [cometary] coma fine structure" may be *ad hoc*.

Second, there is photogeological evidence that the Reiner Gamma swirl on western Oceanus Procellarum is related in origin to a nearby $3.2 \pm 0.2 \times 10^3$ -Myr-old primary impact crater³ and that some of the larger swirls in Mare Ingenii are associated with the Copernican ($1-2 \times 10^3$ Myr old) crater O'Day⁷. Thus, a genetic relationship of all the swirls to the late Copernican crater Goddard A caused by a "prograde, incoming split comet system impacting the Moon" as proposed in ref. 1 is arguable. Note also that although there is evidence that many of the more conspicuous far side swirls are very young, it is not known whether the young swirls are magnetized.

Third, if both the swirls and the high-amplitude magnetic anomalies are caused by comet impacts, magnetic signatures of more ancient (and more numerous) such impacts should have been detected all over the Moon. Instead, virtually all the high-amplitude magnetic anomalies

detected between $\pm 30^\circ$ of latitude with the Apollo subsatellite magnetometers and charged particle instruments occur in limited selenographic regions where swirl patterns exist. The restricted occurrence of both swirls and high-amplitude magnetic anomalies within particular regions suggests a geological rather than an entirely exogenic control on their origin.

Fourth, the distribution of the largest concentrations of swirl patterns (and magnetic anomalies) on the moon is not random as expected for unusual additions of cometary matter. Specifically, as first pointed out by El-Baz⁸, the most conspicuous concentrations of swirls (north of Mare Marginis and within Mare Ingenii) are roughly antipodal to the two youngest large impact basins, Orientale and Imbrium, respectively. As evidence that the antipodal relationship is not fortuitous, the two additional regional concentrations of swirls mapped in Fig. 3b of ref. 1 (centred at 165°W , 23°S and 125°W , 20°S) are within 5° of the Serenitatis and Crisium antipodes, respectively. Each of these four areas is known to be anomalously magnetic^{9,10} and at least three of the four areas is marked by the general occurrence of unusual hilly and lineated terrain^{11,12}. The latter has been ascribed either to compression at the antipode by body and surface seismic waves generated by the associated basin impacts¹³ or to convergence of basin secondaries at the antipode¹⁴. To explain the concentrations of both swirls and large magnetic anomalies near basin antipodes, a compositional modification of the crust in areas where the hilly and lineated terrain is found has been postulated¹⁵ such that subsequent (meteoroid) impacts on these areas result in crater ejecta which is especially susceptible to magnetization. The necessary compositional modification was proposed to be an increase in metallic iron content, as metallic iron is the ferromagnetic carrier in all sampled lunar materials.

L. L. HOOD

Lunar and Planetary Laboratory,
University of Arizona,
Tucson, Arizona 85721

7. Schultz, P. H. *Moon Morphology*, 420 (University of Texas Press, Austin, 1976).
8. El-Baz, F. *Trans. Am. Geophys. Un. (EOS)* **52**, 856 (1971).
9. Lin, R. P., El-Baz, F., Hood, L. L., Runcorn, S. K. & Schultz, P. H. in *Lunar Planet. Sci. XI*, 626-627 (Lunar and Planetary Institute, Houston, 1980).
10. Hood, L. L., Russell, C. T. & Coleman, P. J. Jr (in preparation).
11. Wilhelms, D. E. & El-Baz, F. *U.S. Geol. Surv. Map*, I-948 (1977).
12. Stuart-Alexander, D. E. *U.S. Geol. Surv. Map*, I-1047 (1978).
13. Schultz, P. H. & Gault, P. E. *The Moon* **12**, 159-177 (1975).
14. Moore, H. J., Hodges, C. A. & Scott, D. H. *Proc. 5th lunar Sci. Conf.*, 71-100 (1974).
15. Hood, L. L. in *Conf. Lunar Highlands Crust*, 72-74 (Lunar and Planetary Institute, Houston, 1979).

SCHULTZ AND SRNKA REPLY—In response to Hood's comments concerning our proposed origin of the bright and dark swirls on the Moon and Mercury, we make the following comments.

First, Hood's proposal that these swirls are strongly magnetized deposits of ejecta from major cratering events is not consistent with available photogeological data. Second, although it has been argued that strongly magnetized material can be generated by an impact event¹⁻³, it is another matter to transport this material in an ordered fashion over long distances so as to create a magnetic anomaly during its deposition; secondary impact processes and local mixing would be expected to randomize the incoming magnetized material and prevent the building of an anomaly. Rather, the ejecta must come to rest and acquire remanence in the presence of a field; this is difficult to accomplish for impact-generated magnetic fields except perhaps for near-rim basin-sized events².

Specific responses to Hood's comments are as follows. First, the existence and possible correlation of medium-amplitude anomalies with the Fra Mauro Formation⁴ (Imbrium ejecta deposits) are irrelevant to discussions of the origin of the swirls, other than to note that there are at least three likely processes responsible for lunar magnetic anomalies: impact-induced magnetization preserved in thick ejecta deposits, remanence acquired in the field of a primordial lunar dynamo, and our proposed cometary magnetization⁵. We stated in our report that all three (and likely more) processes have occurred on the Moon during its evolution, but that only cometary magnetization is consistent with the nature of the swirls.

Second, photogeological data do not show that Reiner Gamma is associated with the crater Cavalerius, contrary to the proposal by Hood *et al.*⁴. The swirls are surficial in character and cross a variety of features including secondary craters from Cavalerius. Cavalerius is sufficiently old for craters as large as 10 km to have superposed its ejecta deposits, excavating

1. Schultz, P. H. & Srnka, L. J. *Nature* **284**, 22-26 (1980).
2. Hood, L. L., Coleman, P. J. Jr & Wilhelms, D. E. *Science* **204**, 53-57 (1979).
3. Hood, L. L., Coleman, P. J. Jr & Wilhelms, D. E. *Proc. 10th lunar planet. Sci. Conf.* 2235-2257 (1979).
4. Anderson, K. A. & Wilhelms, D. E. *Earth planet. Sci. Lett.* **46**, 107-112 (1979).
5. Strangway, D. W., Gose, W., Pearce, G. & McConnell, R. K. *Nature* **246**, 112-114 (1973).
6. Fuller, M., Cisowski, S. M. & Hale, C. J. *Proc. 10th lunar planet. Sci. Conf.*, 2211-2233 (1979).

darker basalts from below. It is crucial to note that such excavations are not present on the Reiner Gamma swirls, indicating that these swirls must be much younger than Cavalerius. Furthermore, the swirls are strong forward light reflectors, in contrast to crater rays, and have a photometric phase dependence unlike any ejecta⁶. Increased forward reflection from lunar soil was observed in areas scoured by the exhaust gases from the Apollo LM descent engine. We point out that the long-term process of impact gardening homogenizes the uppermost regolith in a relatively short time, so that such a photometric effect should have disappeared in the Reiner Gamma Formation if it is an old feature. Finally, a small crater on the rim of O'Day (not the older crater O'Day) was suggested as a possible impact site related to the Ingenii swirls; relation of these swirls to the Goddard-A swirls as part of a single cometary impact event is unimportant for our proposed origin.

Third, Hood comments that if the high-amplitude magnetic anomalies are due to cometary impacts, similar features should be common because presumably many comet impacts have occurred. This is incorrect, as our proposed process affects only the upper regolith (<1 m); magnetization there will be lost comparatively quickly due to impact gardening, and viscous decay⁷ of high-field remanence in the altered soil. Thus, only the most recent events, and only those associated with fresh, active comets would be observable as magnetic anomalies.

Fourth, we agree that the apparent connections between swirls, magnetic anomalies and antipodal points of some major basin-forming impacts are intriguing, but we must point out the serious difficulties encountered if these correlations are interpreted as genetically linked. First, the swirls near the crater Goddard A are not at the antipodal point of the Orientale basin, but extend 1,000 km to the east. Hilly and lineated terrains, which may be the result of focused seismic energy⁸ or convergence of secondary ejecta⁹, occur at the antipodes of Imbrium and Orientale; the swirls do not. The claimed correlation of swirl positions with the Crisium and Serenitatis antipodes misrepresents the data, as swirls occur in broad bands which pass through these antipodes⁵. We have mapped swirls in these bands where photography permitted their positive identification, but south of this region the photography is extremely poor; the existence of swirls elsewhere on the Moon cannot be excluded. Finally, note that there is no reasonable candidate basin antipodal to Reiner Gamma.

Thus, we reiterate that lunar magnetization is probably the result of a variety of processes. However, the morphology and photometric properties of the light and dark swirls require both their recent origin and confinement to the

regolith. They are very young features; this obviates the need to invoke magnetic shielding¹⁰ to preserve the high albedo of their bright components.

P. H. SCHULTZ
L. J. SRNKA*

*Lunar and Planetary Institute,
3303 NASA Road One,
Houston, Texas 77058*

* Permanent address: Exxon Production Research Co., PO Box 2189, Houston, Texas 7701.

1. Hide, R. *The Moon* **4**, 39 (1972).
2. Srnka, L. J. *Proc. 8th lunar Sci. Conf.*, 785-792 (1977).
3. Srnka, L. J. et al. *Earth planet. Sci. Lett.* **42**, 127-137 (1979).
4. Hood, L. L., Coleman, P. J. Jr & Wilhelms, D. E. *Proc. 10th lunar planet. Sci. Conf.*, 2235-2257 (1979).
5. Schultz, P. H. & Srnka, L. J. *Nature* **284**, 22-26 (1980).
6. Schultz, P. H., Srnka, L. J., Pai, S. I. & Menon, S. *Lunar Planetary Science XI*, 1009-1011 (Lunar and Planetary Institute, Houston, 1980).
7. Tsay, F.-D. *Nature* **246**, 76-78 (1973).
8. Schultz, P. H. & Gault, D. E. *The Moon* **12**, 159-177 (1975).
9. Moore, H. J., Hodges, C. A. & Scott, D. H. *Proc. 5th lunar Sci. Conf.*, 71-100 (1974).
10. Hood, L. L. & Schubert, G. *Science* **208**, 49-51 (1980).

A ?braidplain facies model for the Westphalian B Coal Measures of north-east England

It was proposed by Haszeldine and Anderton¹ that prominent sand bodies in the Westphalian B of north-east England formed as braided river sheet sands following uplift of local sediment source areas. Our studies of uppermost Westphalian A and Westphalian B sediments in the same region support differing conclusions concerning (1) the extent and significance of palaeocurrent variation, (2) petrographic differences between individual sandstones and (3) petrographic evidence that much of the sediment is recycled.

(1) We agree that successive major sandstones have remarkably diverse and sometimes opposed general palaeocurrent trends. However, we find that individual major sandstones often have widely dispersed palaeocurrents at single outcrops and over large areas have complex patterns. Such variability seems contrary to the concepts of Haszeldine and Anderton, in which braided rivers flow down tectonically tilted palaeoslopes.

The same extremely diverse palaeocurrent trends also often apply to sandstones of the interbedded 'coal bearing facies', some of which probably accumulated in the highest energy environment of small shallow water deltas. Minor divergence could be due to crevassing and to differing orientations of sub-deltas with regard to palaeoslope, but probably not the extremes present.

We suggest that much of this palaeocurrent variability, in the major sandstones and interbedded coal bearing

facies, results from minor structural and palaeotopographic control of depositional systems on a coastal plain of almost non-existent palaeoslope. Such conditions pertain over the broad expanse of the modern Mississippi delta plain (through no analogy of delta style is suggested), where river and distributary channels, and delta lobes have diverse orientations with regard to regional palaeoslope^{2,3}. Subsidence and continued accumulation of similar deposits should produce the sort of complex sediment body architecture present in the uppermost Westphalian A and Westphalian B of County Durham and elsewhere⁴.

(2) We have examined thin sections cut from samples of major sandstones (impregnated with coloured epoxy resin) from County Durham and coastal Northumberland. Varieties and proportions of quartz, feldspars, rock fragments and heavy minerals show few differences that cannot be attributed to grain size^{5,6}, diagenetic dissolution and replacement^{7,8}, problems of readily distinguishing small grains of untwinned feldspar from quartz and the presence of intraformational sedimentary rock fragments. The results of our point counting do not substantiate the petrographic differences between major sandstones that might be expected if they were derived from a number of differing source areas.

(3) Only a small proportion of sand within the major sandstones seems to be recycled, direct evidence for recycling being anomalous grain size-rounding relationships, grains with multiple dust rims and overgrowths, half round grains and the presence of extraformational sedimentary rock fragments^{6,9,10}. Relatively fresh feldspars, including sodic plagioclases, forming on average 10% of framework grains, are hardly suggestive of recycling from low relief sedimentary source areas in humid tropical climates^{6,9,11,12}, neither is the original subangular nature of some framework grains. Our preliminary conclusions suggest derivation of these sandstones from a mixed source area containing granitic, low grade metamorphic and sedimentary lithologies. Devonian sediments are probably indicated by occasional rounded recycled grains with haematite dust rims underlying multiple and abraded overgrowths.

Although we welcome the stimulus to Coal Measures sedimentology provided by Haszeldine and Anderton's report, we urge caution over the application of generalized facies models the the Coal Measures, when in many areas of the UK detailed sedimentological knowledge is limited. We intentionally do not provide an alternative model for the major sand bodies within the Westphalian B of north-east England, as each sandstone requires individual consideration, in the context of its surrounding strata. Our experience in County Durham and in other British

coalfields also suggests that major sandstones, apparently traceable over wide areas, may have accumulated in a number of depositional environments.

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ALAN P. HEWARD
CHRIS R. FIELDING

Department of Geological Sciences,
University of Durham,
Durham DH1 3LE, UK

1. Haszeldine, R. S. & Anderton, R. *Nature* **284**, 51–53 (1980).
2. Fisk, H. N. *Mississippi River Comm.*, Vicksburg (1944).
3. Meckel, L. D. *Bull. Am. Ass. Petrol. Geol.* **56**, 639 (1972).
4. Visser, G. S., Ekebafe, S. B. & Rennison, J. *Deltas, Models for Exploration*, 381–397 (Houston geol. Soc., 1975).
5. Odom, I. E., Doe, T. W. & Dott, R. H. *J. sedim. Petrol.* **46**, 862–870 (1976).
6. Blatt, H., Middleton, G. V. & Murray, R. C. *Origin of Sedimentary Rocks*, 2nd edn (1980).
7. Heald, M. T. & Larese, R. E. *J. sedim. Petrol.* **43**, 458–460 (1973).
8. Wilson, M. D. & Pittman, E. D. *J. sedim. Petrol.* **47**, 3–31 (1977).
9. Folk, R. L. *Petrology of Sedimentary Rocks* (Hemphill, Austin, Texas, 1974).
10. Krynnine, P. D. *Bull. geol. Soc. Am.* **53**, 1850–1851 (1942).
11. Krynnine, P. D. *Am. J. Sci.* **29**, 353–363 (1935).
12. Potter, P. E. *J. Geol.* **86**, 423–449 (1978).

HASZELDINE AND ANDERTON REPLY—Heward and Fielding have highlighted some of the difficulties encountered when attempting to develop a model for the English Westphalian. Our model¹ was not intended as a definitive explanation for the details of the north-east coalfield succession, but as an alternative stratigraphic and sedimentological framework to the 'large delta' model. We proposed a low relief 'coastal plain' depositional environment crossed by many rivers. Heward and Fielding do not disagree with this general idea, but dispute some of the detailed evidence.

Deductions from measured palaeocurrents of the major fluvial facies must be made cautiously, as a wide spread of cross-bedding orientations is expected from any type of fluvial system^{2–4}. Only the highest stage indicators, formed when currents are not influenced by lower stage component flows and channel-floor relief, give an accurate measure of the overall river direction^{3,4}. Even these high stage current directions, fossilized by bar platform or supraplatform stratification, depend on the type of bar in the river and the method of its accretion to the floodplain^{2–4}. Consequently, even from the deposits of one migrating low-sinuosity river, high stage palaeocurrents may easily vary by $\pm 60^\circ$ from the mean river orientation. These palaeocurrents will still, however, show a vector mean roughly downriver, unless accretion to one bank is dominant⁴. Coal Measures washout directions show good agreement with measured high stage palaeocurrent

means, have consistent trends over large areas and have different trends from cycle to cycle. Subsidence resulting from compaction or local faulting may well have produced local palaeolopes, but these were superimposed on the regional drainage pattern. We would expect the sandstones of the coal facies to show a very great palaeocurrent variability, as lakes were probably filled from many directions (ref. 1, Fig. 1).

The sandstones examined by us contain many direct examples of recycled grains. Obviously not all recycled grains will preserve such direct evidence⁵. The apparent lack of extraformational sediment clasts is common in many modern recycled sands⁵. Note also that large quantities of fresh feldspar can survive more than one sediment cycle, and long transport distances in rapidly eroded tropical climates^{6,7}.

As Heward and Fielding suggest, the differences in sandstone mineralogy are not compelling in isolation, but we think that a greater degree of uniformity would be expected of sands derived from one source. Until a large-scale petrographic examination, combined with an appreciation of the relationships between petrography, the sub-environments within a river and the overall depositional environment shows otherwise, mineralogical differences and grain-size differences between sandstones (such as the anomalous coarse-grained sandstone above the Bensham seam in Northumberland) must suggest heterogeneous sediment sources.

We do not imply that all this sediment was supplied from adjacent areas, and would consider such areas as Ireland, the present North Sea and the Caledonides also contributed significantly. Any other more distant source would not account for the increasing marine influence, decrease in sediment grain size and change in river type from Northumberland towards the thickest Coal Measures in the Southern Pennine coalfields. Palaeocurrent trends of major sandstones from the east, west, south-west and north are thus in accord with our model and the overall palaeogeographical context⁸.

R. S. HASZELDINE
R. ANDERTON

Department of Applied Geology,
University of Strathclyde,
Glasgow G1 1XJ, UK

1. Haszeldine, R. S. & Anderton, R. *Nature* **284**, 51–53 (1980).
2. Bluck, B. J. *Scott. J. Geol.* **7**, 93–138 (1971).
3. Bluck, B. J. *Trans. R. Soc. Edinburgh* **70**, 181–221 (1979).
4. Bluck, B. J. *Trans. R. Soc. Edinburgh* **69**, 425–456 (1976).
5. Blatt, H., Middleton, G. V. & Murray, R. C. *Origin of Sedimentary Rocks* (Prentice-Hall, Englewood Cliffs, 1972).
6. Krynnine, P. D. *Am. J. Sci.* **29**, 353–363 (1935).
7. Milliman, J. D., Summerhayes, C. P. & Barretto, H. T. *Geol. Soc. Am. Bull.* **86**, 610–614 (1975).
8. Anderton, R. et al. *A Dynamic Stratigraphy of the British Isles* (Allen & Unwin, London, 1979).

High sediment yields from major rivers of the western Southern Alps, New Zealand

GRIFFITHS has recently estimated¹ erosion rates in the Southern Alps of New Zealand. His presentation, however, takes too little heed of published data, and has under-reported the suspended load data available.

(1) Griffiths reports suspended sediment yields from four east-coast and 10 west-coast rivers. In contrast, I have given yields^{2,3} for 28 east-coast and 18 west-coast rivers. Thompson and Adams⁴ have given yields from 23 of the same east-coast rivers and discussed their calculation and results in detail, and I have given yields⁵ from 40 North Island rivers that have comparably high yields.

(2) Griffiths applies a traditional approach to his calculation of suspended loads. The water flow records presently available are generally good, and are quite adequate for suspended load calculations. However, I have expressed some reservations about the suspended load concentration data^{4,5}. For flows less than the mean, the data are numerous (see Fig. 2 of ref. 1), but inconsequential as their contribution to the long-term mean load is slight. It is unfortunate that the sampling of flood flows is inadequate. Taking the Haast River data¹ as an example, there are 16 samples taken at less than mean flow, but only six at flows greater than the mean.

For many South Island rivers, half the suspended load is carried at flows greater than 3–8 times mean flow⁴. Because the highest flow sampled for the Haast River is only about twice the mean, considerable extrapolation of any concentration–flow relationship is required to calculate loads. Griffiths seems to have used linear regression for the relation, an approach Thompson and I rejected because of the nature of the data. Our reasons and an alternative method are given elsewhere^{4,5}. This method gave the 'earlier crude estimates' mentioned by Griffiths¹.

For all rivers except the Cleddau (see below) estimates by our method average 70% higher than Griffiths'. As there are no independent measurements of load in these rivers (note that both Griffiths and I used essentially the same data), and because estimates of load from point concentration samples may be in error by +100% and –50% (ref. 6), the importance of the difference is uncertain.

(3) I do not know on what additional data Griffiths' estimate of 13,000 tonnes $\text{km}^{-2} \text{yr}^{-1}$ for the Cleddau is based, as I^{2,3} estimated 275 tonnes $\text{km}^{-2} \text{yr}^{-1}$ from concentration and flow data available up to 1977, and showed that the rate of sedimentation in adjacent Milford Sound represented erosion of about 200 tonnes $\text{km}^{-2} \text{yr}^{-1}$. This discrepancy of a factor of 50 is remarkable and needs to be resolved.

(4) Griffiths comments briefly on the

importance of bed load. I have derived^{2,3} a bed load transport formula, checked it against some measured transport rates in New Zealand rivers, and applied it to many South Island rivers. The value of this (as of any bed load equation) is open to question, but it does estimate the bed load of the Waimakariri River as given by Griffiths⁷ to within 15%. Dissolved loads have also been estimated in a general way^{2,3} and, taken with the bed and suspended loads, suggest that for the Southern Alps the suspended load is 93%, the bed load 3%, and the dissolved load 4% of total river load.

(5) Griffiths neglects to mention that sampled river loads need not represent long-term average erosion rates. Some sparse New Zealand data^{2,3} and observations in New Guinea⁸ led me to conclude that in the northern part of the South Island, infrequent, earthquake-caused landslips could double the short-term erosion rate when averaged over hundreds of years.

(6) The obvious relationship between erosion rate and rainfall in the Southern Alps has been widely recognized. My thesis² contains data and discussion, showing that Fournier's⁹ 2.65 power relationship between erosion rate and rainfall fits the estimates of erosion rate of the eastern slopes of the Southern Alps acceptably well. In contrast to the catchment averages used by Griffiths, the integral of local values of erosion and rainfall rate were used⁴. McSaveney¹⁰ deduced a 2.92 power relationship and observed "rainfall is overwhelmingly the dominant influence on present erosion rates", a result later shown by Griffiths' multiple regression analysis.

(7) Griffiths' work is in general agreement with the published work cited above, and with the exception of the value for the Cleddau, I consider his estimates reasonable. There is an overall balance between rates of crustal shortening, tectonic uplift, river erosion, and deposition offshore for the South Island of New Zealand that indicates contemporary uplift and erosion of the Southern Alps⁵. The balance implies long-term erosion rates of up to 48,000 tonnes km⁻² yr⁻¹, perhaps half of which occurs as catastrophic landslips. There seems little doubt that quite large catchments in the Southern Alps presently have erosion rates greater than 15,000 tonnes km⁻² yr⁻¹.

JOHN ADAMS

2-71 James Street, Ottawa,
K1R 5M2, Canada

5. Adams, J. J. *Hydrol. N.Z.* **18**, 36-48 (1979).
6. Walling, D. E. *Erosion and Solid Matter Transport in Inland Waters*, 34 (IAHS Symposium, Paris 1977).
7. Griffiths, G. A. J. *Hydrol. N.Z.* **18**, 6-28 (1979).
9. Pain, C. F. & Bowler, J. M. Z. *Geomorph.* **18**, 92-104 (1973).
9. Fournier, F. *Climat et Erosion* (Presses Universitaires de France, 1960).
10. McSaveney, M. J. *Proc. Conf. Erosion Assessment and Control in New Zealand*, 7-24 (1978).

Griffiths REPLIES—In considering Adams' points (1) and (2) the relevant work is that of Thompson and Adams¹. These authors assert that most of the scatter in New Zealand suspended sediment concentration versus water discharge ratings results from sediment exhaustion effects. They specifically assume that for a given river discharge the concentration on the rising stage of a flood is always higher than on the falling stage. Thompson and Adams¹ support this with results² from a single flood on Mararoa River. Because most concentration data have been collected on the falling stage, the authors compensate for sediment exhaustion by fitting straight lines "drawn midway between the scatter of the points" so as to give equal weight to the limited amount of rising stage data. Among the more serious deficiencies in the justification and application of this methodology are: first, on nearby Shotover River¹ scatter limits in the suspended sediment concentration rating are controlled mainly by seasonal effects. Rising and falling stage data are fairly evenly distributed (D. M. Hicks, personal communication). Second, Thompson and Adams did not identify the latter data. On Waimakariri and Forks Rivers, for example, all measurements were made on falling flood stages or at low flows: yet the ratings still are drawn midway through the scatter of the points¹. Third, Thompson and Adams¹ do not always follow their rating definition prescription. For instance, on Acheron, Ahuriri, Clarence and Rakaia Rivers their ratings do not bisect the data scatter.

Thompson and Adams have made little use of the data: the positions of their rating lines are arbitrary; their results are not repeatable. Consequently, in this context, the relevant works^{1,3} were not considered as serious references. At some sites, however, the rating definition is not too sensitive and there is the stated agreement of results⁵.

Referring to his point (3) for Cleddau River suspended sediment rating, Adams uses a rating line slope of 1.7, without justification³, whereas at his other 45 sites he assumes a value of 2.3. Even using his rating equation I cannot duplicate his yield of 275 tonnes km⁻² yr⁻¹: instead I obtain some 2,000 tonnes km⁻² yr⁻¹, which reduces the discrepancy to a factor of 6.5. The residual discrepancy is accounted for by his arbitrary definition of the suspended sediment rating. Similar wide discrepancies arise at other sites for the same reason. For example, in

Mangatu, Waingaromia and Waipaoa Rivers, Adams⁵ lists yields of 28,000, 20,000, 14,000 tonnes km⁻² yr⁻¹ respectively, whereas I calculate, using the same data, values of 7,000, 17,000, 5,000 tonnes km⁻² yr⁻¹. Adams⁵ supports his Cleddau River yield estimate with sedimentation data obtained from a single borehole in Milford Sound. He assumes suspended sediment deposition, age and areal extent of deposit and size of contributing area. This does not, in my view, constitute adequate scientific evidence.

My comments on points (4) and (5) would be too lengthy; my conclusions are similar to point (3).

With regard to point (6) Fournier's⁶ relationship as applied by Thompson and Adams¹ does not fit the estimates of erosion rate acceptably well. In fact, the constant E_0 , in their modified equation varies from 2,000 to 160,000 tonnes km⁻² yr⁻¹ (see Table 1 in ref. 1).

On Adams' point (7), McSaveney (personal communication) estimates a long-term yield in excess of 50,000 tonnes km⁻² yr⁻¹ in Cropp River, a tributary of Hokitika River⁵. Erosion in this catchment occurs by natural fluvial processes including frequent landslips under high intensity storms.

Finally, the units of suspended sediment concentration used in Fig. 2 and in the calculation of the coefficients in my first paragraph⁵ are given incorrectly as kg m⁻³ instead of g m⁻³. This error, however, does not enter into the results or conclusions.

GEORGE A. GRIFFITHS

Water and Soil Division,
Ministry of Works and Development,
PO Box 1479, Christchurch, New Zealand

1. Thompson, S. M. & Adams, J. *Physical Hydrology—New Zealand Experience* (eds Murray, D. L. & Ackroyd, P.) 213-229 (New Zealand Hydrological Society, Wellington, 1979).
2. Christian, R. & Thompson, S. M. J. *Hydrol. N.Z.* **17**, 50-53 (1978).
3. Adams, J. thesis, Victoria Univ. Wellington (1978).
4. Adams, J. J. *Hydrol. N.Z.* **18**, 36-48 (1979).
5. Griffiths, G. A. *Nature* **282**, 61-63 (1979).
6. Fournier, F. *Climat et Erosion* (Presses Universitaires de France, 1960).

Molecular clockwork

THE results of the very interesting experiment of Hartl and Dykhuizen¹ can be interpreted differently. There are three statistical problems with their conclusion that response by *Escherichia* to natural selection occurs as rapidly with generations of 5 h as with those of 2.5 h.

First, in their Table 2, basing an analysis on the last 200 h instead of the last 300, as equally justified by their own argument on the duration of the later periodic selection, would reverse the conclusion, the rate of evolution being much greater in the

1. Griffiths, G. A. *Nature* **282**, 61-63 (1979).
2. Adams, J. thesis, Victoria Univ., Wellington (1978).
3. Adams, J. *Bull. geol. Soc. Am.* **91**, 2-4; 1-114 (1980).
4. Thompson, S. M. & Adams, J. *Physical Hydrology—New Zealand Experience* (eds Murray, D. L. & Ackroyd, P.) 213-229 (New Zealand Hydrological Society, Wellington, 1979).

chemostats with shorter generation time. Second, non-significant differences are taken to be zero in averaging in Table 2. Using all information (ignoring significance because zero differences in fitness are not really expected and 10 of 11 non-significant differences are ostensibly positive, as expected) gives mean fitness increments of 1.78 per 100 h for shorter-period chemostats and 1.18 for longer-period ones, using the last 300 h, and 2.33 and 0.55, respectively, using the last 200 h. Again, faster evolution with more rapid generations is suggested.

Third, in competition between the same strains grown in each of the two kinds of chemostats, interstrain selection coefficients in both conditions can be compared (their Fig. 2). The trend line summarizing the results seems, however, to be an ordinary regression rather than a co-regression², even though the two variables depend equally on each other and there is no asymmetrical prediction involved. A co-regression would be steeper and thus suggest that elimination of weaker strains is more rapid per unit time with short generations than with longer generations; even the regression given is significant in this direction at the 0.05 level. One might want to modify the statistics of their Table 2 by this result, but alternatively, it can itself be taken as another indication of faster change with faster turnover. I do not want to imply that the results suggest a proportionality between generation number and evolutionary rate, merely a positive relationship.

Conceptually, the experiment is interpreted (even in the title) as showing that a constant evolutionary rate per unit time can be caused by natural selection. However, even if the data gave clear support to the conclusions drawn (and I think they suggest the opposite), what would be shown is that generation time does not influence evolutionary rate even under strong selection pressure. The rates of change observed were far from constant even though (perhaps because only three time intervals are involved) not significantly more uneven than rates of molecular evolution. There is no evidence to suggest a long-term approximate constancy in mean rate, as there is for molecular evolution, and an independence of evolutionary rate and generation time has long been advocated³ from palaeontological evidence. Such an independence, to the extent it exists, is easily explained if evolutionary rate is caused largely by changes in the total environment. A stochastically constant rate of evolution is a much more difficult phenomenon to explain. This gives it power in a genetically based view of evolution; only neutralism predicts it more or less adequately in that context. However, if we take ecology seriously with respect to evolutionary change, a natural (but still unproved) explanation

for the observed degree of constancy emerges⁴. It is quite consistent with the observed large short-term differences⁵⁻⁷ in evolutionary rates, as neutralism may not be⁷.

LEIGH M. VAN VALEN

*Biology Department (Whitman),
University of Chicago,
915 East 57th Street,
Chicago, Illinois 60637*

1. Hartl, D. & Dykhuizen, D. *Nature* **281**, 230-231 (1979).
2. Van Valen, L. *J. theor. Biol.* **45**, 235-247 (1974).
3. Simpson, G. G. *The Major Features of Evolution* (Columbia University Press, New York, 1953).
4. Van Valen, L. *J. molec. Evol.* **3**, 89-101 (1974).
5. Kurtén, B. *Cold Spring Harb. Symp. quant. Biol.* **24**, 205-215 (1960).
6. Van Valen, L. *Evol. Theory* **4**, 45-80 (1978).
7. Lessios, H. A. *Nature* **280**, 599-601 (1979).

DYKHUIZEN AND HARTL REPLY—

Before discussing the particular points raised by Van Valen, we wish to emphasize that the molecular clock is sloppy¹, so exactitude should not be expected. It would be wonderful to carry out such experiments for 500 years (or, better yet, 500 Myr). In such a long time span, different results might very well be obtained. We have achieved only 500 h. Nevertheless, our data have remarkable internal consistency for experiments of this sort, and the formal congruence between the characteristics of our results and of the molecular clock are too striking to ignore. Also, we do not suggest that the environment is unimportant to the rate of evolution. The chemostat is a novel environment for *E. coli* K2, and it is likely that the large initial selection is due to this change of environment. It is also possible that the average rate of selection would decrease over time in the constant environment of the chemostat. Chemostats could be run for 2,500 h to see if this supposition is true. If true, it implies that environmental change is required to keep the selective clock wound.

Van Valen has questioned why we chose to analyse the last 300 h rather than the last 200 h. Most of our argument is actually based on the full 500 h as evidenced by the statement "The overall [that is, 500 h] average fitness increment per 100 h is 2.99 in the 2.5 h chemostat and 3.00 h in the 5.0 h chemostat"². (Incidentally, if one treats all fitness differences as if they were significant, as Van Valen suggests, the corresponding averages are 3.28 and 3.05.) We presented the averages for the period 200-500 h only to show that we were not being misled by the large fitness increments that occur in the first 200 h. Van Valen points out that if only the last 200 h are taken for analysis, then it looks as if the 2.5 h strain is evolving faster. But this ignores more than half of the experimental measurements, and it contradicts the conclusion from the entire data set. By using a selected subset of any data, one can contradict any conclusion whatsoever.

There is, however, a better way to settle the issue, which is by direct competition between strains that have evolved for long periods at either a 2.5-h or a 5.0-h generation time. For strains that had evolved for the full 500 h, the largest fitness difference is observed when the experimental test is carried out in a 2.5-h chemostat; the 2.5-h strain is favoured, to be sure, but the fitness difference is a mere 5%. This amount of genetic divergence over 500 h is very small indeed. Moreover, when tests are carried out in a 5.0-h chemostat, selection actually favours the 5.0-h strain. These observations are difficult to reconcile with a presumed more rapid rate of evolution at a 2.5-h generation time³.

The data in our² Fig. 2 are not relevant to the issue of rate of increase in fitness; they pertain to whether measured selection coefficients depend on the conditions of measurements. If the slope of the line in Fig. 2 is truly greater than 1.0, as Van Valen suggests, then we have actually overestimated the rate of increase in fitness in 2.5-h chemostats by about 25%, so our original conclusion receives additional support. Moreover, Van Valen's suggestion of co-regression is well taken. The co-regression line⁴ has a slope of 1.5, which, if taken literally, calls for a correction of about 35%, so our original conclusion is more forcefully justified by Van Valen's own technique.

In short, our results indicate that the rate of increase in fitness is related to the amount of time spent in the chemostat rather than the number of generations. The issue is clearly an important one, and we hope that this exchange with Van Valen will encourage more of such experiments to be undertaken.

D. DYKHUIZEN
D. HARTL

*Purdue University,
Department of Biological Sciences,
West Lafayette, Indiana 47907.*

1. Fitch, W. M. in *Molecular Evolution* (ed Ayala, F. J.) 160-178 (Sinauer, Sunderland, Massachusetts, 1976).
2. Hartl, D. & Dykhuizen, D. *Nature* **281**, 230-231 (1979).
3. Dykhuizen, D. & Hartl, D. *Evolution* (in the press).
4. Sokal, R. and Rolf, F. J. *Biometry* 526-532 (Freeman, San Francisco, 1969).

Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

BOOK REVIEWS

Certainty into doubt: 25 years of nuclear debate

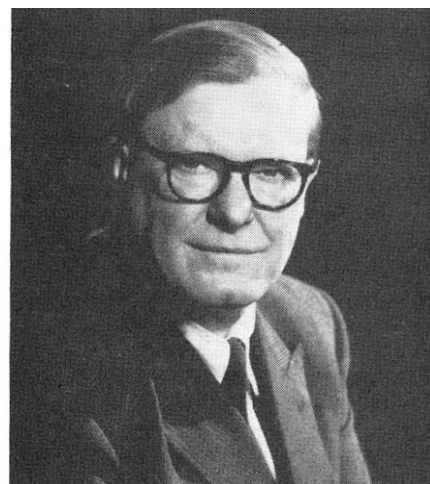
Alan Cottrell

The Nuclear Power Decisions: British Policies, 1953–78. By Roger Williams. Pp.365. (Croom Helm: London, 1980.) £19.95. Available from Biblio Distribution Centre, Totowa, New Jersey, \$49.

THERE IS a saying in the nuclear industry that the reactor you choose is always the best one. It becomes the best one because you work on it, solve its problems, improve it, develop and adapt a whole technology to it, all of which gives it an edge over its rivals. There are certainly grains of truth in this, but the irony is that different countries have different 'best' reactors and hang on to their choices grimly through thick and thin. Today we have each of the three original reactor-producing countries firmly entrenched in its own concepts of thermal reactors: the gas-cooled types, Magnox and AGR, for the British; the light-water ones, PWR and BWR, for the Americans; and the heavy-water one, CANDU, for the Canadians.

It is interesting to follow these deep, insurmountable, trenches back in time to their earliest beginnings in the 1940s as slight traces across the smooth face of the then infant nuclear era. There were the Americans using light-water cooling in their early Hanford reactors, choosing it for its simplicity and because they had the empty spaces in which to site these possibly dangerous plants. From this early background they went on to light-water pressurized reactors for propelling submarines, out of which grew the modern pressurized-water and boiling-water civil reactors. Again there were the British, with their lack of large rivers for coolant in very remote and unpopulated regions, choosing gas-cooling for their early Windscale military reactors, which likewise set them firmly in their own groove; and yet again there were the Canadians, keen to make the best use of natural uranium and so being led to heavy-water, to which they became equally heavily addicted. Only the French, in switching from gas cooling to light-water cooling, dared to jump over into another trench.

Much of this is brought out in Roger Williams's splendid book, *The Nuclear Power Decisions*, which discusses the major British decisions in the civil nuclear field, and the way they were made, between 1953 and 1978. Mostly, the book deals with the history of the Advanced Gas-cooled Reactor (AGR) — of how, despite its various imperfections and vicissitudes, it



Sir John Cockcroft (left) and Lord Penney — "heroes" of the 1950s and 60s.

successfully fought off all threats from every water-cooled rival, basically because it was a gas-graphite reactor, a British concept with British technology and expertise, a direct descendant of the Windscale, Calder Hall and Magnox reactors. Williams takes the reader carefully and clearly through the history of this, giving full weight to all the technical, economic, political and, not least, personal factors involved in the decisions. The sound of those old debates of the 1950s and 60s, now half-forgotten, comes thundering back with the reminders of the great speeches by the heroes of that period — Hinton, Penney, Cockcroft, Plowden, Sherfield, Coleraine, Stanley Brown and others; as well as the clash of argument between the AEA and the CEBG. We are also reminded of the amazing surefootedness of that early period, of the launching in the mid-1950s of a huge programme of Magnox reactors even though their prototype, Calder Hall, was still one year away from completion. Contrast that with the snail-like approach we are making today towards the Fast Breeder Reactor. How did we lose our nerve? Williams is too polite to say but he does make a vigorous plea in his final chapter for a system of public accountability that would make civil nuclear energy as acceptable to the general public in the 1980s as it so obviously was in the 1950s.

Perhaps the difficulties of the AGR had something to do with it, causing us to doubt whether our 'best' really was the best of bests. Williams takes the reader through the dense jungle of AGR politics with immense skill and clarity, especially in regard to the economic and political

factors. Despite the intentionally non-technical nature of the book, more could perhaps have been made of the technical differences between AGR and the water reactors, and their significance, particularly from the point of view of safety, for these factors do have important weight in the decision-making. Nevertheless, the account is a very fair and well-balanced one, which is no mean feat in a field where emotions run so high that one distraught civil servant felt impelled to complain that it was impossible for anyone to remain impartial where the choice of reactors is concerned.

The final section of the book deals with the 1978 Windscale decision. This is not only a change of subject — the expansion of the nuclear reprocessing plant — but even more a reflection of the change of mood since the 1950s. No longer is there the gung-ho approach of the early White Papers on reactor programmes. Instead we are in the era of doubt, of public enquiries, of Mr Benn's hesitations, of the cautionary remarks of the Flowers Report, and of the campaigns by the Friends of the Earth and other anti-nuclear groups. So much has the native hue of nuclear resolution been sicklied o'er by the pale cast of thought. And this leads to Williams's final chapter on public accountability and his "conviction that Britain can have a much more successful industrial future than she has had an immediate past, especially where nuclear power is concerned". A book strongly to be recommended. □

Sir Alan Cottrell is Master of Jesus College, University of Cambridge.

Advances in research on evoked potentials

D. Regan

Evoked Potentials. Edited by C. Barber. Pp. 614. (MTP Press: Lancaster, UK, 1980.) £29. *Clinical Uses of Cerebral, Brainstem and Spinal Somatosensory Evoked Potentials.* Edited by J.E. Desmedt. Pp. 354. (Karger: Basel, 1980.) SFr 138, \$82.75. *Cognitive Components in Cerebral Event-Related Potentials and Selective Attention.* Edited by J.E. Desmedt. Pp. 322. (Karger: Basel, 1979.) SFr 118, \$70.75.

IT HAS been known for almost 100 years that the human brain continuously generates an electrical oscillation called the electroencephalogram (EEG) that can be recorded from electrodes held onto the scalp. The EEG can reach an amplitude of 100 μ V, so that the brain's 0.1–10 μ V responses to auditory, visual, tactile or olfactory stimulation are usually hidden in the EEG. Consequently, these electrical responses to sensory stimulation, called evoked potentials, were almost unexplored until automatic signal-processing devices, developed in the 1950s, were used to separate evoked potentials from the brain's background activity. Part A of Barber's book is an excellent, concise survey of these signal-processing techniques and evoked potential methodology.

The three books reviewed here together cover all the main directions of current evoked potential research. Many contributors emphasize the present clinical value and future promise of evoked potential recording as an effectively risk-free means of objectively testing hearing, vision and spinal cord function. Clearly, a battery of objective sensory tests would meet a need, particularly with neonates and young infants, where the available methods for testing sensory function are imprecise. In adults, evoked potential tests can distinguish between organic and psychogenic disorders, and practical successes with adult patients are described by authors in all three books. In particular, short-latency evoked potentials recorded from auditory brainstem structures have proved their worth both in the neurological clinic and in assessing hearing disorders, and evoked potentials produced by visual patterns are widely used as an aid to diagnosing multiple sclerosis. Auditory brainstem responses are a promising means of testing infants' hearing, and a technically difficult method for recording from electrodes over the spinal cord has been successfully used with infant patients, but so far visual evoked potentials seem to have had comparatively little impact on the visual testing of infants and the management of amblyopia. Part A of Barber's

book identifies limitations in our understanding of visual evoked potential generation that must be overcome if the method is to fulfil its exciting promise in paediatric neuro-ophthalmology.

Evoked potential recording is the only widely available, risk-free means of objectively exploring the activities of normal human brains (analysis of the brain's magnetic field is still at an early stage of development). Consequently, evoked potential recording has a special appeal to scientists interested in aspects of brain activity that may be peculiar to the human species. Rather than studying evoked potentials generated by sensory stimuli, a second line of research explores scalp potentials produced or modified by thinking processes. Several authors in Desmedt's book on cognition show how evoked potentials can be used not only to study the brain activities associated with mental evaluation of incoming sensory information, but also how to study cognitive aspects of brain function including selective attention.

All three books are collections of contributions by different authors, each of whom focuses chiefly on his own research area. Although this style of book can be assembled more quickly and with less individual toil than is the case when the writing is undertaken by only one or two authors, there are disadvantages for the reader. These include greater difficulty in following a coherent thread through successive chapters, and some unevenness in the standard of different authors' contributions. Since each of the books edited by Desmedt addresses a restricted topic these drawbacks are less evident than in Barber's book. Desmedt's two books provide researchers and clinicians with summaries of current research in cognitive evoked potential studies and in somatosensory evoked potentials that are as useful as any available. However, it is a

pity that substantial linking commentaries were not incorporated, since these would have made both books more effective as introductory texts. Although its inclusion may have delayed publication, the edited discussion on pattern responses in Barber's book adds considerably to the value of the text. Several more such discussions could have been added with advantage.

Barber's book is based on the 1978 evoked potential conference held in Nottingham, and resembles in some ways a book based on the 1974 Brussels conference (*Visual Evoked Potentials in Man*. Edited by J.E. Desmedt. Clarendon: Oxford, 1977). Some impression of progress in evoked potentials research can be obtained by comparing these two volumes. What emerges is that the relation between cognition and long-latency evoked potentials is more complex than was thought to be the case in 1974; it now seems that several late waves exist, differing from one another in their brain origins and psychological correlates. Our basic understanding of visual evoked potentials advanced substantially before 1974, but progress seems to have slowed since then. The few researchers attempting to develop evoked potentials as much-needed objective measures in psychiatry have continued to encounter formidable problems including the broadness of clinical diagnostic classifications. In contrast, the use of visual evoked potentials in the neurology clinic, a research technique in 1974, is now routine in a large number of hospitals. Perhaps the major new thrusts in basic research and in clinical applications have been in auditory brainstem and somatosensory evoked potentials, and these topics are well covered both by Barber and by Desmedt. □

D. Regan is I.W. Killam Research Professor in the Department of Physiology and Professor of Ophthalmology at Dalhousie University, Halifax, Nova Scotia.

Protein synthesis from start to finish

P. J. Ford

From DNA to Protein: The Transfer of Genetic Information. By M. Szekely. Pp. 284. (Macmillan Press: London, 1980.) Hardback £18, \$35.95; paperback £9.95.

From DNA to Protein is an up-to-date (early 1979) account of that fascinating but ill-defined area of biology comprising some genetics, some biochemistry and some macromolecular structure. The book has been written for advanced undergraduates and postgraduate students, but is also intended for research workers wishing to keep up with molecular biology.

My initial reaction to the contents pages was very favourable since the book is logically ordered, starting with a

description of DNA structure and replication, followed by transcription and messenger RNA structure and leading into a discussion of translation. There is also a chapter on methods of nucleotide sequence determination which, for some undergraduate teaching purposes, might be too specialized. Such detailed attention nevertheless does indicate the overwhelming role of technology in forcing advances in this area, a point which I think cannot be made too often.

Reading the book in more detail I found that descriptions of particular systems were generally easily followed although there are many places where more careful editing would have improved communication. Some more obvious errors might also have been removed such as the "thousands (of ribosomal RNA genes) in *Drosophila*" (p. 16) or more seriously the suggestions that satellite DNAs are small molecules (p. 28) and that yeast has a single

chromosome (p. 34).

On p. 41 there is the ambiguous statement that "In higher organisms in which the enzyme patterns of the different cells are fairly constant, the protein genes are not part of operons. They are dispersed in the chromosome, transcribed separately and their expression is in most cases individually controlled". This is the point at which the author comes nearest to discussing the fundamental problem in eukaryotic gene regulation, which is how an apparently constant genome manages to produce such a diversity of cellular structure and function during development and differentiation. However, the theme of coordinate gene regulation in eukaryotes is not pursued. Nor is any attempt made to rationalize known genetic linkage maps in terms of gene regulation. The passage also serves to illustrate a general weakness of the text, which shows a preoccupation with minutiae and failure to state basic problems

clearly and extract a useful synthesis of ideas from the welter of information. In particular I had expected a discussion of gene structure on the basis of the discoveries of overlapping and split genes, since this is promised on the dust cover. Although overlapping and split genes are mentioned on several separate occasions, there is no single passage which attempts to draw together all the observations and to deal with their consequences for the control of messenger RNA synthesis and for the evolution of protein structures.

Some specific topics which I would have liked to see mentioned are transposition and insertion elements as model mechanisms for moving DNA within and between chromosomes; a more detailed treatment of lambda phage as a model for switching between two alternate (lytic or lysogenic) types of development; a treatment of gene numbers in terms of rate limits to transcription; consideration of

immunoglobulin genes in relation to specific reorganization of DNA during differentiation; and some discussion of secretion which involves a hydrophobic amino-terminal signal peptide enabling proteins for secretion to cross membranes.

For students the reviews of DNA replication, transcription and translation should be useful and are well referenced up to 1978. Although these chapters relate almost exclusively to prokaryote systems they do give comparisons with eukaryote translation mechanisms, eukaryote RNA polymerases and eukaryote DNA replication mechanisms. The other chapters on the structure of messenger RNA, the structure and function of ribosomes and on transfer RNA are perhaps too specialized for all but the devotee of macromolecular structure and function. □

P. J. Ford is a Lecturer in the Department of Molecular Biology at the University of Edinburgh.

1937, 1951, 1976 — progress in crystallography

D.W.J. Cruickshank

X-Ray Analysis and the Structure of Organic Molecules. By Jack Dunitz. Pp. 514. (Cornell University Press: 1980.) \$55, £36.

THIS BOOK has developed out of the Baker Lectures given by Jack Dunitz at Cornell University in the fall of 1976. Once again the publication of a set of the Lectures allows them to be appreciated by a far wider audience than the chemists of Cornell. This volume immediately brings to mind Linus Pauling's influential Lectures of 1937, "The Nature of the Chemical Bond and the Structure of Molecules and Crystals", and J.M. Robertson's notable contribution of 1951, "Organic Crystals and Molecules". Crystallography and organic chemistry have developed enormously in the last quarter century, so that whereas in 1951 Robertson could discuss a large proportion of the 200 or so organic crystal structures then known, in 1976 it would have been impossible for Dunitz to survey 16,000 structures in like fashion.

Dunitz's book is in two parts: "Crystal Structure Analysis" and "Molecular Structure". The first part is a fairly complete guide to current methods of determining the crystal structures of small or medium-size molecules (not proteins). A score or two of books must have been written on crystal structure analysis, but Dunitz's volume is more attractive than most of the others. This is because of the flowing informal style and the penetrating insights and asides of a master of the craft. I suspect his

treatment will prove somewhat too professional for most of those chemists he wants to help read the crystallographic literature, but it will be invaluable for young (or not-so-young) crystallographers seeking an overview of the main parts of the subject.

Among the interesting features is a discussion of the frequency of occurrence of particular space groups and Kitaigorodsky's explanation of this in terms of possible molecular packing arrangements. On p.136 we learn of the caesium salt of boromycin, the absolute configuration of which was established before its structural formula. On p.143 we read of the flurry caused in 1972 by Tanaka's claim that all absolute configurations determined by X-ray analysis were wrong and should be changed into their antipodes. Tanaka's claim proved ill-founded, but his error was a happy one for it led to new and independent proofs of the correctness of the Bijvoet method. There is a helpful and well-titled section on hazards of oblique coordinate systems. Both this and the section on thermal motion analysis contain useful worked examples.

Unfortunately the treatment of Fourier transforms is marred by a misprint at a critical point on p.30, and by a failure to make clear whether the inverse transform relates to the minus sign in the exponential argument or to the transformation from **R** space to **r** space. Dunitz is a Lewis Carroll fan and he concludes his summary of simple theorems about Fourier transforms with a quotation from *Alice*:

"What is the use of repeating all that stuff", the Mock Turtle interrupted, "if you don't explain as you go along? It's by far the most confusing thing I have ever heard!"

Dunitz must have had a premonition of trouble on the printed page!

The second part on molecular structure contains four chapters, of which the first, "Crystal Structure Analysis and Chemistry", is both the most substantial and the one of greatest general interest. Dunitz first traces the explosive growth of information on molecular structures and stresses the utility of the computer-based files of structural data collected by the Cambridge Crystallographic Data Centre. He reminds us of the triumphs of classical organic chemistry in the determination of structural formulae, but uses patchouli alcohol as an example of an unexpected contradiction. He reports on conformational analysis, solid-state chemistry and reaction intermediates — the latter illustrated by the remarkable structure of $\text{CO}_2(\text{CO})_4(\text{H}_2\text{CBu})(\text{C}_2\text{H}_5)$ determined by Mills and Robinson in 1964. Then comes a discussion of molecular potential energy surfaces, which leads on to Dunitz's structural correlation principle whereby the numerous slightly differing 'still' pictures of the same structural fragment in different crystals are used to infer a chemical reaction path. Nucleophilic addition to carbonyl groups is used as one of the examples of this principle.

The remaining chapters include a good account of investigations into the electron-density distributions in molecules, a very clear exposition of the geometrical aspects of the closure constraints in cyclic molecules and an elegant discussion of symmetry in conformational maps.

Dunitz learnt his crystallography in Glasgow and Pasadena. Robertson and Pauling can be well satisfied with the volume their former protégé has now placed alongside their own in the Baker Lecture series. □

D.W.J. Cruickshank is Professor of Chemistry (Theoretical) at the University of Manchester Institute of Science and Technology, and was formerly General Secretary and Treasurer of the International Union of Crystallography.

BOOKS RECEIVED

Mathematics

- BEAUMONT, G. P. *Intermediate Mathematical Statistics*. Pp. 248. Flexi ISBN 0-412-15480-3. (Chapman & Hall: 1980.) flexi £5.95.
- CHAKRAVARTI, I. M. (ed.). *Asymptotic Theory of Statistical Tests and Estimation*. The Proceedings of an Advanced International Symposium held at the University of North Carolina, April, 1979. Pp. 350. ISBN 0-12-166650-6. (Academic: 1980.) \$25.
- KINDERLEHRER, D. and STAMPACCHIA, G. *An Introduction to Variational Inequalities and Their Applications*. Pure and Applied Mathematics Series. Pp. 313. ISBN 0-12-407350-6. (Academic: 1980.) \$35.
- MATSUMURA, H. *Commutative Algebra*. 2nd Edn. Mathematics Lecture Note Series. Pp. 312. Flexi ISBN 0-8053-7026-9. (Benjamin/Cummings: Reading, Massachusetts, 1980.) \$19.50.
- SNEDDON, I. N. *Special Functions of Mathematical Physics and Chemistry*. 3rd Edn. Longman Mathematical Texts. Pp. 182. Flexi ISBN 0-582-44396-2. (Longman: 1980.) £5.95.
- WILSON, A. G. and KIRKBY, M. J. *Mathematics for Geographers and Planners*. 2nd Edn. Pp. 408. Hbk ISBN 0-19-874114-6; pbk ISBN 0-19-874115-4. (Clarendon/Oxford University Press: 1980.) Hbk £12.50; pbk £6.95.

Astronomy

- BATRICK, B. and MORT, J. (compiled by). *Proceedings of the 2nd European IUE Conference*, Tübingen, Germany, March 1980. Pp. 368. Flexi ISBN 0379-6566. (European Space Agency: Paris, 1980.) F.Frs. 125.
- BURKE, W. L. *Spacetime, Geometry, Cosmology*. Pp. 329. ISBN 0-935702-10-6. (University Science Books: Mill Valley, California, 1980.) NP.
- BURKE, W. R. *Photovoltaic Generators in Space*. Proceedings of the 2nd European Symposium on the Photovoltaic Generators in Space, Heidelberg, Germany, April 1980. Pp. 249. Flexi ISBN 0379-6566. (European Space Agency: Paris, 1980.) F.Frs. 80.
- DIXON, R. T. *Dynamic Astronomy*. 3rd Edn. Pp. 524. Flexi ISBN 0-13-221267-6. (Prentice/Hall: 1980.) £9.70.
- DOHERTY, P. *Atlas of the Planets*. Pp. 143. ISBN 0-600-30439-6. (Hamlyn: Feltham, UK, 1980.) £6.95.
- LEVITT, I. M. and MARSHALL, R. K. *Star Maps for Beginners. A Fireside Book*. Pp. 64. Flexi ISBN 0-671-25422-7. (Simon and Schuster: New York, 1980.) NP.
- WALKER, J. (introduction by). *Light from the Sky. Readings from Scientific American*. Pp. 78. Flexi ISBN 0-7167-1222-9; hbk ISBN 0-7167-1221-0. (W. H. Freeman: 1980.) Flexi £3.30; hbk £7.10.
- GRIBBIN, J. *The Strangest Star. A Scientific Account of the Life and Death of the Sun*. Pp. 184. ISBN 0-485-11207-8. (Athlone: London, 1980.) £6.95.

Applied Biology

- BATTISTIN, L., HASHIM, G. and LAJTHA, A. (eds). *Neurochemistry and Clinical Neurology. Progress in Clinical and Biological Research Vol. 39*. Pp. 500. ISBN 0-8451-0039-4. (Alan R. Liss: New York, 1980.) \$38. Available in Europe and the Middle East from European Book Service, Flevoalaan 36-38, P.O.B. 124, 1380 AC Weesp, Holland.
- BOON, M. E. and TABBERS-BOUMEESTER, M. L. *Gynaecological Cytology. A Textbook and Atlas*. Pp. 192 + 144 atlas. Hbk ISBN 0-133-26117-8; flexi ISBN 0-133-26117-6. (Macmillan: London, 1980.) Hbk £25; flexi £14.
- BRENNAN, M. J., McGRATH, C. M. and RICH, M. A. (eds). *Breast Cancer: New Concepts in Etiology and Control*. Pp. 406. ISBN 0-12-131150-3. (Academic: 1980.) \$31.50.
- BRIDGES, J. W. and CHASSEAUD, L. F. (eds). *Progress in Drug Metabolism Vol. 4*. Pp. 333. ISBN 0-471-27702-9. (Wiley-Interscience: 1980.) £25.
- CALABRESE, E. J. *Nutrition and Environmental Health. The Influence of Nutritional Status on Pollutant Toxicity and Carcinogenicity. Vol. 1 The Vitamins*. Pp. 585. ISBN 0-471-04833-X. (Wiley-Interscience: 1980.) £32.70.
- CIBA FOUNDATION SYMPOSIUM 74 (New series). *Drug Concentrations in Neuropsychiatry*. Pp. 266. ISBN 90-219-4080-9. (Excerpta Medica: 1980.) \$45.75, Dfl. 94.
- CONDORELLI, L., TEODORI, U., ANGIUSSOLA, A. B. and SANGIORGI, M. (eds). *Internal Medicine. Proceedings of the XIV International Congress of Internal Medicine ISIM Rome, October 1978. Parts 1 and 2*. Pp. 1532. ISBN 90-219-0438-1. (Excerpta Medica: 1980.) Parts 1 and 2 \$195, Dfl. 400.
- COON, M. J. *et al.* (eds). *Microsomes, Drug Oxidations, and Chemical Carcinogenesis Vol. 4th International Symposium on Microsomes and Drug Oxidations held at Ann Arbor, Michigan, July 1979*. Pp. 618. ISBN 0-12-187701-9. (Academic: 1980.) \$39.50.
- COON, M. J. *et al.* (eds). *Microsomes, Drug Oxidations, and Chemical Carcinogenesis Vol. II. 4th International Symposium on Microsomes and Drug Oxidations held at Ann Arbor, Michigan, July 1979*. Pp. 1258. ISBN 0-12-187702-7. (Academic: 1980.) \$41.
- COUNCIL FOR INTERNATIONAL ORGANIZATIONS OF MEDICAL SCIENCES (CIOMS). *International Nomenclature of Diseases. Vol. III, Diseases of the Lower Respiratory Tract*. Pp. 128. Flexi ISBN 92-9036-001-1. (CIOMS: Geneva, 1979.) Sw.fr. 25.
- DANIELS, S., HALSEY, M. J. and SMITH, E. B. (eds). *Techniques for Diving Deeper than 1,500 feet. UMS Publication No. 40 WS (DD) 6-30-80. The 23rd Undersea Medical Society Workshop*. Pp. 159. (Undersea Medical Society: 9550 Rockville Pike, Bethesda, Maryland 20014, 1980.) Pbk np.
- DE BOER, J. and BAILLIE, T. W. (eds). *Disasters: Medical Organization*. Pp. 110. ISBN 0-08-025491-8. (Pergamon: 1980.) £6.50, \$14.50.
- DOSMAN, J. A. and COTTON, D. J. (eds). *Occupational Pulmonary Disease; Focus on Grain Dust and Health. Proceedings of the International Symposium on Grain Dust and Health held in Saskatoon, Canada, November 1977*. Pp. 615. ISBN 0-12-221240-1. (Academic: 1980.) \$42.
- ENGLISH, M. P. *Medical Mycology*. Pp. 57. Flexi ISBN 0-7131-2795-3. (Edward Arnold: London, 1980.) £2.50.
- FAGLIA, G., GIOVANELLI, M. A. and MACLEOD, R. M. (eds). *Pituitary Microadenomas. Proceedings of the Sero Symposium, Vol. 29*. Pp. 566. ISBN 0-12-248150-X (Academic: 1980.) £30, \$69.
- FRASER, F. C. *et al.* *Le Diagnostic Pérénal. Cahiers de Bioéthique Vol. 2*. Pp. 281. Flexi ISBN 2-7637-6914-4. (Les Presses de L'Université Laval, Quebec: 1980.) \$14.
- GIORDANO, C. (ed.). *Sorbents and Their Clinical Applications*. Pp. 503. ISBN 0-12-285280-8. (Academic: 1980.) \$55.
- GLENNER, G., COSTA, P. E. and de Freitas, A. F. (eds). *Amyloid and Amyloidosis. Proceedings of the Third International Symposium on Amyloidosis, Póvoa de Varzim, Portugal, September 1979*. Pp. 629. ISBN 90-219-0426-8. (Excerpta Medica: 1980.) \$109.75, Dfl. 225.
- GUTHRIE, F. E. and PERRY, J. J. (eds). *Introduction to Environmental Toxicology*. Pp. 484. ISBN 0-632-00659-5. (Blackwell Scientific: 1980.) £16.50.

- HAERER, A. F. (ed.). *Self-assessment of Current Knowledge in Neurology*. 3rd Edn. Pp. 226. (Medical Examination Publishing: New York, 1980.) \$17.
- HERSON, A. C. and HULLAND, E. D. *Canned Foods. Thermal Processing and Microbiology*. 7th Edn. Pp. 380. ISBN 0-443-02122-8. (Churchill Livingstone: 1980.) £14.
- HUFSTADT, A. F. C. *Congenital Malformations. The Jönxis Lectures. A series of post-graduate medical courses. Vol. 4*. Pp. 360. ISBN 90-219-6004-4. (Excerpta Medica: 1980.) \$44, Dfl. 90.
- INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS. *Microbial Ecology of Foods. Vol. 1. Factors Affecting Life and Death of Microorganisms*. Pp. 332. ISBN 0-12-363501-2. (Academic: 1980.) \$29.50.
- JENNER, P. and TESTA, B. (eds). *Concepts in Drug Metabolism Part A. Drugs and the Pharmaceutical Sciences Vol. 10*. Pp. 109. ISBN 0-8247-6906-6. (Dekker: 1980.) Sw.fr. 98.
- KLEIN, G. and WEINHOUSE, S. (eds). *Advances in Cancer Research Vol. 32-1980*. Pp. 361. ISBN 0-12-006632-7. (Academic: 1980.) \$36.
- LATNER, A. L. and SCHWARTZ, M. K. (eds). *Advances in Clinical Chemistry Vol. 21*. Pp. 254. ISBN 0-12-01321-4. (Academic: 1980.) \$29.
- LEIGH, H. and REISER, M. F. *The Patient. Biological, Psychological, and Social Dimensions of Medical Practice*. Pp. 351. ISBN 0-306-40207-6. (Plenum Medical: 1980.) \$19.50.
- LEIGH, J. H. *The Timber Trade: An Introduction to Commercial Aspects*. 2nd Edn. Pp. 115. Flexi ISBN 0-08-024916-7; hbk ISBN 0-08-024917-5. (Pergamon: 1980.) Flexi £4.20, \$9.50; hbk £8, \$18.
- LEUNG, A. Y. *Encyclopedia of Common Natural Ingredients Used in Food, Drugs and Cosmetics*. Pp. 409. ISBN 0-471-04954-9. (Wiley-Interscience: 1980.) £26.60.
- LEVITT, P. M. and GURALNICK, E. S. *The Cancer Reference Book. Direct and Clear Answers to Everyone's Questions*. Pp. 271. ISBN 0-440-51353-7. (Delta Books, Dell Publishing Co. Inc. 1 Dag Hammarskjöld Plaza; 245 East 47 Street, New York, N.Y. 10017:1980.) Flexi \$4.95, \$5.75 Canada.
- OLTON, D. S. and NOONBERG, A. R. *Biofeedback. Clinical Applications in Behavioral Medicine*. Pp. 437. ISBN 0-13-076315-2. (Prentice/Hall: 1980.) £13.65.
- OOTA, K., MAKINODAN, T., IRIKI, M. and BAKER, L. S. (eds). *Aging Phenomena; Relationships among Different Levels of Organization. Advances in Experimental Medicine and Biology Vol. 129*. Pp. 317. ISBN 0-306-40460-5. (Plenum: 1980.) £37.50.
- OWEN, O. S. *Natural Resource Conservation; An Ecological Approach*. 3rd Edn. Pp. 883. ISBN 0-02-390020-2. (Collier Macmillan: 1980.) £12.95.
- PEBERDY, J. F. *Development Microbiology*. Pp. 230. Hbk ISBN 0-216-91018; pbk ISBN 0-216-91018-8; pbk ISBN 0-216-91019-6. (Blackie: 1980.) Hbk £17.50; pbk £8.50.
- PRESTAKO, A. W., CROOKE, S. T. and CARTER, S. K. (eds). *Cisplatin; Current Status and New Developments. Papers presented at a symposium sponsored by the University of Alabama in Birmingham Comprehensive Cancer Centre and Bristol Laboratories September 1979, in Atlanta, Ga.* Pp. 527. ISBN 0-12-565050-7. (Academic: 1980.) \$30.
- RAINS, D. W., VALENTINE, R. C. and HOLLAENDER, A. (eds). *Genetic Engineering of Osmoregulation; Impact on Plant Productivity for Food, Chemicals, and Energy. Basic Life Sciences Vol. 14*. Pp. 381. ISBN 0-306-40454-0. (Plenum: 1980.) \$39.50.
- RICHTER, G. W. and EPSTEIN, M. A. *International Review of Experimental Pathology Vol. 21-1980*. Pp. 303. ISBN 0-12-364921-8. (Academic: 1980.) \$34.50.
- ROBERTS, P. J. (ed.). *Biochemistry of Dementia. Based on a Workshop on Biochemistry of the Dementias, held at University of Southampton, March 1979*. Pp. 272. ISBN 0-471-27698-7. (Wiley-Interscience: 1980.) £16.
- ROSSOF, A. H. and ROBINSON, W. A. (eds). *Lithium Effects on Granulopoiesis and Immune Function. Advances in Experimental Medicine and Biology Vol. 127. Proceedings of the Workshop held at Rush University, Wisconsin, June 1979*. Pp. 475. ISBN 0-306-40359-5. (Plenum: 1980.) \$47.50.

Psychology

- ARONSON, E. *The Social Animal*. 3rd Edn. Pp. 377. Flexi ISBN 0-7167-1230-X; Hbk ISBN 0-7167-1229-6. (W. H. Freeman: 1980.) Flexi £4.30, Hbk £9.50.
- CRAIG, G. J. *Human Development*. Pp. 560. ISBN 0-13-444984-3. (Prentice/Hall: 1980.) £11.65.
- HARDIN, G. *Promethean Ethics; Living with Death, Competition, and Triage*. Pp. 82. ISBN 0-295-95717-4. (University of Washington Press: 1980.) \$7.95, £4.80.
- PERRET-CLERMONT, A. N. *Social Interaction and Cognitive Development in Children. European Monographs in Social Psychology 19*. Pp. 206. ISBN 0-12-551950-8. (Academic: 1980.) £14.80, \$34.
- TURNER, R. R. and REESE, H. W. (eds). *Life-Span Developmental Psychology. Intervention*. Pp. 346. ISBN 0-12-704150-8. (Academic: 1980.) \$24.50.

General

- DEPARTMENT OF NATIONAL DEFENCE. *Defence 1979*. Pp. 118. Flexi ISBN 0-660-50455-3. (Department of National Defence: Ottawa, Canada, 1980.) Flexi \$3 Canada; other countries \$3.60.
- DRINKWATER, P. and SELF, E. *A Passion for Garlic*. Pp. 128. Hbk ISBN 0-7156-1404-5; pbk ISBN 0-7156-1405-3. (Duckworth: London, 1980.) NP.
- HERBERT, S. *The Red Notebook of Charles Darwin*. Pp. 164. Bulletin of the British Museum (Nat. Hist.) Historical Ser. Vol. 7. Pbk ISSN 0068-2306; hbk ISBN 0-8014-1226-9. (British Museum (Natural History)/Cornell University Press: 1980.) Pbk £9; hbk £10.
- LASZLO, E. and KURTZMAN, J. (eds). *The Structure of the World Economy and Prospects for a New International Economic Order. Pergamon Policy Studies on the New International Economic Order*. Pp. 118. ISBN 0-08-125119-6. (Pergamon: 1980.) £7.50, \$15.
- MOHTADI, M. F. (ed.). *Man and His Environment Vol. 3. Proceedings of the Third International Banff Conference on Man and His Environment held in the Banff Springs Hotel, May, 1978*. Pp. 152. ISBN 0-08-025792-5. (Pergamon: 1980.) \$37, £16.50.
- MURPHY, E. F. *Energy and Environmental Balance. Pergamon Policy Studies on Energy and Environment*. Pp. 281. ISBN 0-08-025082-3. (Pergamon: 1980.) £16.25.
- TALMOR, E. *Descartes and Hume*. Pp. 174. ISBN 0-08-024274-X. (Pergamon: 1980.) \$7, £7.50.
- URQUHART, J. *Living off Nature*. Pp. 396. ISBN 0-7139-1229-4. (Allen Lane: London, 1980.) £7.95.
- WHITLOCK, R. *Rare Breeds: The Vulnerable Survivors*. Pp. 160. ISBN 0-904727-87-4. (Prism: Dorchester, UK, 1980.) £8.95.
- WORLD HEALTH ORGANIZATION. *The Work of WHO, 1978-1979. Biennial Report of the Director-General*. Pp. 264. Flexi ISBN 92-4-156063-0. (World Health Organization: Geneva, 1980.) Flexi SwFr. 18. Also available in Arabic, Chinese, French, Russian and Spanish.